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Munich, June 15, 2005

Europäisches Patentamt

80298 München

Opposition against:	EP 0656786 (93909679.8)
Proprietor:	Novogen Research Pty Ltd.
Opponent:	Wolfgang Richter
Our Ref.:	J100703EP MR/ADK/sd

OPPOSITION

Opposed patent:	Patent No. EP 0 656 786 B1
Application No.:	93909679.8 (PCT/AU1993/000230)
Date of filing:	May 19, 1993
Priority:	May 19, 1992 (AU 2511992)
Date of Publication and mention of the grant of the patent:	September 15, 2004
Title:	"*Use of Isoflavone Phyto-Oestrogen Extracts of Soy or Clover*"
Patentee:	Novogen Research Pty Ltd. 140 Wicks Road North Ryde, NSW 2113, Australia

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The official fee of € 610.00 is to debited from deposit account no. 28 001 197.

Requests

1. Revocation of EP 0 656 786 B1 in its entirety is requested.

2. Oral proceedings are requested, if request 1 will not be granted.

I. Grounds for Opposition

The opposition is filed on the grounds of Art. 100 (a) EPC because the subject matter of the opposed patent is not patentable under Art. 54 and 56 EPC.

The opposition is also filed on the grounds of Art 100(b) EPC, because the patent does not disclose the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art (violation of Art 83 EPC).

Finally, the opposition is also filed on the grounds of Art 100 (c) EPC because the patent contains subject matter which extends beyond the content of the application as filed and violates Art 123 EPC.

The opponent relies on the following documents which will be referred to by the numbers given below:

D1: Virgil's Online Guide to Fighting Prostate Cancer (http://www.prostate-online.com/wnew9808.html.

D2: WO 94/23716 (referred to as D14 in the examination proceedings (letter of applicant's representative from July 21, 2003) including priority document and print out from EPO online register).

D3: JP 61246124 A, abstract (referred to as D2 in the examination proceedings (letter of applicant's representative from July 21, 2003)).

D4: JP 01258669A, abstract (referred to as D26 in the examination proceedings (letter of applicant's representative from July 21, 2003)).

D5: Wie funktioniert das? Der Mensch und seine Krankheiten, Bibliographisches Institut, Mannheim 1984, page 540.

D6: Kaldas and Hughes, "Reproductive and general metabolic effects of phytoestrogens in mammals", Reproductive Toxicology Review, 1989, pages 81- 89 (referred to as D32 in the examination proceedings (letter of applicant's representative from July 21, 2003)).

D7: JP 62106016 A (abstract) (referred to as D4 in the examination proceedings (letter of applicant's representative from July 21, 2003)).

D8: Adlercreutz et al, "Dietary phyto-oestrogens and the menopause in Japan", The Lancet, May 16 1992, Vol 339 (referred to as D14 in the examination proceedings (letter of applicant's representative from July 21, 2003)).

D9: Adlercreutz et al., J., "Dietary phytoestrogens and cancer: in vitro and in vivo studies", Steroid Biochem. Molc. Biol. 1992, Vol 41, No 3-8, pages 331-337.

D10: Wong, J. Sci Food Agric. 1962, Vol 13, pages 304-307 (referred to as D18 in the examination proceedings (letter of applicant's representative from July 21, 2003).

D11: Tamura et al., Agr. Biol. Chem., Vol. 33, pages 391-397, 1969 (referred to as D19 in the examination proceedings (letter of applicant's representative from July 21, 2003).

D12: Wong and Flux, "The oestrogenic activity of red clover isoflavones and some of their degradation products ", J. Endorcin., 1962, Vol 24, pages 341-348

D13: Puffe et al, Das wirtschaftseigene Futter, 1984, Vol 30, No 3, pages 184-201.

D14: Adlercreutz, "Western Diet And Western Diseases - Some Hormonal And Biochemical-Mechanisms And Associations", Scandinavian Journal of Clinical & Laboratory Investigation, 1990, 50, Suppl 201, pages 3-23

D15: Lark Susan M., "The Menopause self-help book", 1990, published by Celestial Arts, Berkeley, California, United States

D16: Adlercreutz et al., "Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet", Am J Clin Nutr. 1991 Dec; Vol. 54 No. 6, pages 1093-100.

D17: Setchell et al., "High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical and thermospray mass spectrometric detection", Journal of Chromatography, (Netherlands) 1987, Vol. 386, pages 315-323.

D18: Peterson and Barnes, "Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation", The Prostate, 22:335-345 (1993).

References D3, D4, D6, D7 to D17 are to be regarded as prior art under Art. 54 (2) EPC, as they were all published before the priority date of the opposed patent.

Reference D2 is to be regarded as prior art under Art. 54 (3) EPC since the priority of the opposed patent is invalid. D2 has a filing date that is earlier than the filing date of the opposed patent and it has been published after the filing date of the opposed patent. A print out from the EPO online register has been attached to D2 to show that the document has been regionalised at the EPO and that the designation fees have been paid.

References D1 and D5 have been submitted to show part of the common general knowledge or for providing additional information.

Almost eleven years passed between the filing of the application and its grant. Seven official actions have been issued during the prosecution phase of the opposed patent. Several sets of amended claims have been filed, as well as, amended pages of the description. The Examiner has only accepted the final set of claims after applicant's representative filed as a response to an official action on October 21, 2003 sworn declarations from the inventor, Graham Edmund Kelly, and from a scientist, Claude L Hughes, explaining an allegedly underlying inventive step of the alleged invention.

As will be demonstrated below, we believe that the arguments provided by applicant's representative, Mr. Kelly, and Mr. Hughes, although well presented, are not supported by the content of the application as filed nor by the patent law under the EPC.

Furthermore, we submit that during the examination phase the description and the claims were changed in an inadmissible way. Furthermore, we believe that the arguments made by the applicant's representative during the examination phase with respect to the priority of the opposed patent claims are not in line with the Decision G2/98 of the last Board of Appeal.
Finally, we submit that there is no actual invention disclosed in the opposed patent. If the disclosure in the application is considered to contain an invention, we submit that the alleged invention is definitely not disclosed in a way that is complete enough for a person skilled in the art to work it.

II. Scope of the Claims of the Opposed Patent

The alleged invention claimed in EP 0 656 786 B1 (hereinafter the "opposed patent") relates to the use of an isoflavone extract of soy or clover for the manufacture of a medicament for the treatment of specific diseases. The patent has only one independent claim (claim 1) and 10 dependent claims (claims 2 to 11). All claims are drafted in second medical-use format.

II.1. Claim 1

Claim 1 has the following features:

1. the use of an isoflavone phyto-oestrogen extract for the manufacture of a medicament for administration,

2. wherein the administration is carried out in unit dosage form,

3a. wherein the extract is that of soy

or

3b. wherein the extract is that of clover,

4a. wherein the medicament is for the treatment of pre-menstrual syndrome or,

4b. wherein the medicament is for the treatment of symptoms associated with menopause

or

4c. wherein the medicament is for the treatment of prostate cancer.

II.2. Claim 2

Claim 2 refers to claim 1 and specifies that the medicament may further comprise at least one excipient suitable for dietary use.

II.3. Claim 3

Claim 3 is dependent on both claim 1 or 2 and specifies that the isoflavone phyto-oestrogen extract is from soya. We note that claim 3 does not provide any additional technical feature over claim 1.

II.4. Claim 4

Claim 4 refers to claim 3 and specifies that the extract is from soya hypocotyls.

II.5. Claim 5

Claim 5 depends on claim 1 or claim 2 and specifies that the extract is from clover. We note that claim 5 does not provide any additional technical feature over claim 1.

II.6. Claim 6

Claim 6 refers to claims 1 to 4 and specifies that the extract comprises one or more of the following: genistein, daidzein or glycosides thereof, or metabolites or derivatives thereof.

II.7. Claim 7

Claim 7 refers to claim 1 or claim 2 and specifies that specific isoflavone phyto-oestrogens are present in the extract in a range of ratios.

II.8. Claim 8

Claim 8 refers to all preceding claims and specifies that the isoflavone phyto-oestrogens are present in a range of amounts per unit dose.

II.9. Claim 9

Claim 9 refers to all preceding claims and specifies that the administration of the medicament is to be carried out at least daily over a period of at least a month. These features relate to non-patentable subject-matter according to Art. 52(4) EPC.

II.10. Claim 10

Claim 10 refers to all preceding claims and specifies that the extract also includes members of three groups of compounds, namely coumestans, lignans and flavones.

II.11. Claim 11

Claim 11 refers to all preceding claims and specifies the unit dosage to be a tablet or a capsule.

III. The subject matter of the opposed patent extends beyond the content of the application as filed (Art. 100 (c); Art. 123 EPC)

III.1. Supplementary pages 8, 8a and 8b

Applicant's representative filed three supplementary pages (supplementary pages 8, 8a and 8b) on February 7, 2001. Although quite a substantial amount of texts were added, applicant's representative had chosen not to indicate precisely where support for the supplemented pages

could be found. Applicant's representative indicated that the supplementary pages serve to adapt the description to the amended claims.

III.1.1. Paragraph 4 of supplemented page 8 contains the feature "unit dosage" with respect to the extract. In our view, this feature is only disclosed in original claim 7 of the application as originally filed. This claim refers back to claim 1 which specifies a health supplement containing a selection of isoflavones. By introducing amended page 8, this feature has been generalised to all isoflavones and is inadmissible, as violating Art 123(2) EPC.

III.1.2. The second paragraph from bottom of page 8 specifies the chemical composition of the extract as being a water/organic solvent extract. In our view, this feature was only disclosed in example 1. However, example 1 only refers to a red clover extract, which is a particular species within the genus clover. The generalisation of an extract from a particular species to an extract of the genus clover is inadmissible. Furthermore, there is no disclosure of a specific soy extract in the application. Therefore, the feature of water/organic solvent extract of soy is inadmissible, as violating Art 123(2) EPC.

III.1.3. The last paragraph on supplemented page 8 lists specific treatments in which the extracts may be used. The application as originally filed only referred to humans as recipients of the food supplement (or arguably the extract). In this respect we refer to, for example, page 8 of the application as originally filed, wherein in the "disclosure of the invention" section, it is stated that

"the present invention concerns a health supplement ... to improve the health of humans."

Amended page 8 no longer contains this limitations and as a result, animals not just humans become potential recipients of the extract. This generalisation is inadmissible as violating Art 123(2) EPC.

III.1.4. The first paragraph on page 8a specifies a treatment regime (daily over one month). Support for this feature can in our view only be found in original claim 17. This claim refers back to claim 10 and discloses this regime only with respect to a health supplement

containing a selection of isoflavones. The generalisation to using all isoflavones is inadmissable.

III.1.5. In the third paragraph on page 8a, the disclosure relating to a specific treatments is not limited to humans (see III.1.3.).

III.2. Claim 1

Claim 1 has been inadmissibly broadened for the same reasons as given already under III.1.3. The claim contains no limitation to treatment of humans although the description only discloses a treatment for humans. The description does not disclose treatment of animals generally.

It is submitted that, for example, dogs also may suffer from prostate cancer (compare D1, page 1 bottom paragraph). The treatment of dogs would also fall under the scope of claim 1 as granted and thus violates Art 123(2) EPC.

III.3. Claims 2 to 11

Claims 2 to 11 depend on claim 1 and are therefore also not in compliance with Art. 123(2) EPC.

IV. The Effective Date of the Claims

EP 0 656 786 B1 claims priority of Australian patent application no. AU 2511992. According to the decision G2/98 of the Enlarged Board of Appeal the priority of a previous application in respect of a claim in a European patent application in accordance with Art 88 EPC is to be acknowledged only if the skilled person can derive subject-matter of the claim directly and unambiguously, using common general knowledge, from the previous application as a whole.

The features of the claims as granted cannot unambiguously and clearly be derived from the priority document AU 2511992.

Claim 1 contains the feature "isoflavone phyto-oestrogen extract of soy or clover". The feature "extract" is only disclosed on page 8 lines 19 to 24 of the priority document:

> *"Similarly, these materials maybe used as a source of coumestans and isoflavins for further chemical or physical extraction."*

"*These materials*" refer to soya hulls only or soya hypocotyls only or a mixture thereof (see priority document, page 8, lines 14 to 19). Thus, the priority document may arguably contain disclosure of an extract of soy hypocotyls or soy hulls but there is definitely no clear and unambiguous disclosure for the feature extract of "soy" in general (which would include other parts of the plant such as soy seeds or cotyledon). This is further highlighted by the fact that the application as filed teaches that it is a further advantage to use the hypocotyl as a source of isoflavones compared to the whole soya bean, because of the relative incidences of the different isoflavones in the hypocotyl as compared to the cotyledon (page 7 third paragraph from top of the application as originally filed).

With respect to clover, the description only mentions subterranean clovers which include a limited number of species belonging to the genus of Trifolium (see priority document, page 9, line 17). However, there is no disclosure of other types of clover. More importantly, there is no clear and unambiguous disclosure at all of extracts of red clover or clover generally.

The features "extract of soy" and "extract of clover" are therefore not entitled to the priority date of AU 2511992.

The technical features of claims 2, 3 and 5 are not entitled to the priority date for the same reasons as above.

Claims 6, 7, 8, 9 and 10 recite technical features of a medicament made with an extract comprising specific isoflavone phyto-oestrogens in specific ratios, specific amounts, or a treat-

ment regime for using the medicament. These features are not disclosed in AU 2511992 and therefore these claims are not entitled to the priority of AU 2511992.

Claims 4 and 11 depend on claim 1 and are therefore not entitled to the priority date either.

Consequently, the effective filing date of the claims as granted is the filing date of the European application, i.e., May 19, 1993.

V. Lack Of Novelty Over D2 (WO 94/23716)

D2 is prior art according to article 54 (3) EPC in connection with article 54 (4) EPC and R23a. D2 discloses and claims the use of an isoflavonoid in the preparation of a medicament for treating or preventing symptoms of menopause or premenstrual symptoms. The invention taught in D2 features a medicament or dietary product comprising isoflavonoids, such as genistein, daidzein, biochanin A and formononetin. In particular, on page 1, line 24 to 28, D2 discloses that isoflavonoids are constituents of soybeans and other plants, and are effective in reducing the symptoms of menopause and pre-menstrual syndrome. On page 2, line 9, it is disclosed that isoflavonoids may be administered in the form of a plant extract (soy extract in particular on page 2, line 16; an isoflavonoid-containing fraction extracted from soy on page 3, lines 1 to 4). D2 explains that soy-based foods are not readily available or palatable to women accustomed to a Western-style diet (D2, page 2, lines 31 to 35). D2 further discloses that the isoflavonoid can be administered in medicament form, for example in the form of a tablet (page 3, lines 30 to 33). Thus, the technical features of claims 1, 2, 3, and 11 are disclosed in D2.

On page 3, lines 14 to 15 of D2, an administration of at least 30 mg per serving is disclosed. Thus, the technical features of claim 8 are disclosed in D2.

On page 3, lines 19 to 24 of D2, a mixture of genistein and daidzein in a ratio of 2:1 as a preferred embodiment is disclosed. Thus, the features of claims 6 and 7 are disclosed in D2.

Claim 9 contains a single technical feature, i.e., a regimen involving administration of the medicament once daily over a period of at least one month. This feature relates to non-commercial and non-industrial medical activities and cannot be used to delimit the claimed subject-matter from the prior art because it relates to subject matter excluded from patentability by Art 52(4) EPC (compare T317/95).

VI. The Opposed Patent Lacks Inventive Step

VI.1. Claim 1 lacks inventive step

This claim relates to the use of an isoflavone phyto-oestrogen extract for the manufacture of a medicament for treatment in unit dosage form of specific symptoms or conditions.

We point out that there is no definition for the term "unit dosage form" in the patent. Consequently, a unit dosage form can be any mode of administration of the medicament, i.e. tablets, capsules, infusions etc. Therefore, the use of an unit dosage form in claim 1 cannot be considered as a solution to the problem presented.

Furthermore, it is emphasized that the patent does not provide any data showing that the administration of an extract of soy or clover actually leads to the remediation of the three conditions recited in claim 1. Therefore, the objective problem to be solved by the invention cannot lie in the treatment of these three conditions. In view of the foregoing, we submit that there is no indication that this problem has been solved by the invention.

Therefore, based on the language of claim 1, the problem that is purported to be solved must be the provision of an alternative source of isoflavones for the manufacture of a medicament. This problem is allegedly solved by using an isoflavone phyto-oestrogen extract of soy or clover. This interpretation is supported by the description in paragraphs [0029], [0030] and [0031] which discuss the difficulty in changing the diet of communities in developed coun-

tries and the strategy of providing phyto-oestrogen in purified form or providing foodstuffs enriched with phyto-oestrogens.

VI.1.1. Lack of inventive step of claim 1 over D3 (use of isoflavone clover extract as medicament)

One of the closest prior art is D3 (JP 61246124A) which discloses the isoflavone, genistein, "separated from subterranean clover" for use as a carcinostatic agent. D3 also teaches the use of various modes of administration and a daily dose of 200 to 1000 mg.

The only difference between D3 and claim 1 is that an isolated isoflavone (genistein) from clover is used instead of an extract of clover. Genistein has been mentioned in the application as filed as a preferred isoflavone (see, for example, claim 6 as granted), so it must be assumed the extract contains genistein.

In paragraph [0030] of the opposed patent, it is stated that an alternative strategy for making available phyto-oestrogens in a purified form is to be considered. We point out that the isolation and purification of a specific compound, such as an isoflavone from plants inevitably involves an extraction step.

Given that it has been suggested that a single isoflavone (genistein) from clover has activity as a medicament, it follows that an extract of the whole clover plant would also contain this isoflavone, and therefore also possess activity useful as a medicament. In other words, it is obvious to use an extract of clover containing isoflavones in the preparation of a medicament when it is known that clover contains isoflavones of medical activity.

There is no data in the patent that shows that an isoflavone phyto-oestrogen extract of clover is in any way superior to the individual isoflavone phyto-oestrogens isolated from clover for manufacturing a medicament.

Therefore, it is obvious for a person skilled in the art who is looking for an alternative source for isoflavones as a medicament to start with D3 and use an isoflavone phyto-oestrogen extract from clover.

VI.1.2. Lack of inventive step of claim 1 over D4 (use of isoflavone soy extract as medicament)

The same argument applies *mutatis mutandis* to the use of an isoflavone phyto-oestrogen extract from soy in view of the teaching of D4. D4 discloses the use of an extract from soybeans for isolating isoflavone compounds that have a range of medicinal properties including oestrogenic activity and anticancer activity. D4 also teaches that the main substances in the extract which have carcinostatic activity are genistein and daidzein, and their derivatives. Therefore, it is obvious for a person skilled in the art who is looking for an alternative source for isoflavones as a medicament to start with D4 and use an isoflavone extract from soy.

VI.1.3. Lack of inventive step of claim 1 in view of D3 (for the feature clover extract) or D4 (for the feature soy extract) combined with D6 or the common general knowledge (for the feature, treatment of prostate cancer).

It has already been stated that the opposed patent does not provide any data for the treatment of prostate cancer. Therefore, the opposed patent does not solve the problem of treating prostate cancer and this feature should be disregarded in the assessment of inventive step. However, even if this feature is taken into consideration, the solution suggested by the opposed patent would lack an inventive step.

Applicant's representative filed sworn declarations by Graham Edmund Kelly, the inventor of the opposed patent; and by C. Hughes, in response to an official action on November 5, 2003. In the Kelly declaration, it was argued in point 6 that

"there is a wide range of human cancers that are well known to be insensitive to drugs that have potent anti-cancer activity against specific forms of cancers... Prostate cancer is a noto-

riously difficult cancer to treat. The only chemotherapeutic approach of any significance is anti-androgen therapy .... Drugs with classical direct cytostatic or cytotoxic effect on cancer cells were typically not used clinically because of lack of efficacy."

We submit that Kelly's statement would indicate that he believed that although it was known that an isoflavone could be used as an agent against cancer, it would not have been obvious for a person skilled in the art to recognize that this agent could also be used as an agent for treating specifically prostate cancer. We believe this statement is misleading for the following reasons:

First, it is submitted that it has been part of the common general knowledge at the priority date that prostate cancer is a hormone-responsive tissue and anti-androgen therapy is used as a treatment of prostate carcinoma as demonstrated by the teachings in the textbook, D5. Since it was known that isoflavones are oestrogenic (see the opposed patent at paragraph [0011]), the use of isoflavones in prostate cancer as part of the anti-androgen therapy of D5 would be obvious.

Furthermore, D6 suggests the use of phytoestrogens for the treatment of cancers of hormone-responsive tissues (page 88, right hand column, first paragraph from top). As demonstrated by D5, the prostate is known to be a hormone-responsive tissue.

Additionally, D9 clearly suggests isoflavones ("Ifls" in D9) as being protective with regard to the development of prostate cancer (page 331, right hand column, lines 15-17).

D14 provides an overview of the associations between diet and sex hormones in the context of Western diseases which include prostate cancer. In a section entitled "Prostate Cancer" on page 14 of D14, in lines 19 to 23 in particular, it is stated that epidemiological data available at the time suggests that

> *"high levels of isoflavonic phytoestrogens in the traditional diet of Japanese men ... may also represent a protective factor ... inhibiting the growth of already existing small cancers."*

D14 further stated that data based on Adventist men and Hawaiian men of Japanese ancestry lent further support to the concept that intake of dietary isoflavones reduce the risk of prostate cancer. D14 concludes by suggesting that dietary isoflavones directly influence cancer cell growth and that the effect of soybean diets on prostate cancer may be a parallel to the observation of the inhibitory effect of soybean diet on breast tumor incidence in experimental animals (see page 14, second column, lines 1-7). We point out that, based on D14, a skilled person in the art at the time of the priority application would have known that dietary isoflavones would likely have an inhibitory activity against more than one type of cancer, that is not just breast cancer but also prostate cancer. This fact is also supported by the opposed patent, for example, in paragraphs [0028] and [0024]:

> *"... it could be reasonably deduced from dietary data that greater levels of foodstuff high in estrogens in the standard diets in developed countries could be expected to redress a general imbalance... thereby reducing the predisposition of those communities to the above diseases."*

The "above diseases" include prostate cancer, menopausal syndromes and pre-menstrual syndromes, see paragraph [0024].

D16, published by the same author further confirmed the association of diet and incidence of prostate cancer by studying the urinary excretion of isoflavone phyto-oestrogens and lignans. D16 also referred to a study which showed the preventive effect of dietary soy in an animal model of prostate cancer.

D18 also suggests using isoflavones for the treatment of human prostate cancer.

Therefore, D3 (with respect to isoflavone extract of clover) or D4 (with respect to the isoflavone extract of soy) combined with the common general knowledge or with D9 or with D6 or with D14 or D18 and the common general knowledge teach all features of claim 1.

VI.1.4. Lack of inventive step of claim 1 in view of D6, D15, D7 combined with D6 (treatment of menopausal or pre-menstrual symptoms).

As already stated above the treatment of the three specific conditions has not been shown to be solved. Therefore, it is to be disregarded for determining the problem to be solved by the claimed invention.

Even assuming that the treatment of menopausal or pre-menstrual symptoms were to be considered as a problem to be solved, the solution suggested by the opposed patent would be obvious for the following reasons:

D6 teaches that phyto-oestrogen isoflavones interfere with oestrogen metabolism. Therefore, their use in the treatment of pre-menstrual and menopausal symptoms is obvious. Moreover, a treatment of menopausal symptoms is explicitly taught in D6 (page 88, right hand second paragraph from bottom). Their use in Chinese herbal medications for the treatment of menopausal symptoms is mentioned. The fact that D6 also discloses that phytoestrogens may have detrimental properties, cannot be construed as teaching away from the invention since the skilled person looking for compounds to be used as medicaments has to take into account potential undesirable side effects.

D7 discloses the use of a genus of isoflavones (genistein, for R1 = OH and R2 = H, biochanin A for R1 = methoxy and R2 = H) for the treatment of osteoporosis. Osteoporosis is one of the symptoms of menopause. Genistein is one of the isoflavones named as a preferred isoflavone and its abundance in clover and soy is well known in the art (see description of the opposed patent).

D15 is a book, first published in 1990, by Dr. Susan M. Lark who used layman's language to explain the use of herbs including red clover to treat the symptoms of menopause. In particular, on pages 131, red clover is disclosed to be useful for treating menorrhagia as well as hot flushes and vasomotor symptoms. She teaches on page 130 that "the herbs should be used in small amounts and taken with your meals either in capsule form or in a tea." Thus, any one, even those without knowledge in the art would have known to use a capsule (which is a type of dosage form) or a tea (which is a form of extract) containing red clover as a medicament for treatment of symptoms of menopause.

Consequently, D6, D15, or D6 combined with D7 teach all features of claim 1.

VI.1.5. Lack of inventive step of claim 1 over D8

The already cited paragraph [0028] of the opposed patent states that

> *"... it could be reasonably deduced from dietary data that greater levels of (foodstuff) high in estrogens in the standard diets in developed countries could be expected to redress a general imbalance... thereby reducing the predisposition of those communities to the above diseases."*

The "above diseases" include prostate cancer, menopausal syndromes and pre-menstrual syndromes, see paragraph [0024]. Here, the opposed patent itself acknowledges that it can be reasonably deduced from such epidemiological data, that greater intake of estrogens may be beneficial for the diseases and symptoms specified in claim 1.

However, the epidemiological data referred to in the opposed patent were not identified. We note these data may be found, for example, in D8, or D14 as discussed above.

It has been known before the filing date of the patent or priority document that isoflavone phyto-estrogens are oestrogenic (see for example D10, page 304; D6, page 81, left hand column; D12, summary section or as admitted in paragraph [0011] of the opposed patent). It is therefore obvious to use an isoflavone phytoestrogen extract in the preparation of a medicament for the treatment of the symptoms and diseases recited in claim 1.

Before the filing date of the patent or priority document it was known that clover and soya contain isoflavone phyto-estrogens and how they can be isolated or concentrated (see for example D10, page 304, top paragraph; D6, page 82; D11, page 391, left hand paragraph; D13, tables 4 to 6; D17; or in paragraphs [0049] and [0055] of the opposed patent). We note that D17 discloses a method for extracting isoflavones from soy and soy products using an aqueous/organic solvent mixture, and involves distillation and drying, prior to high performance liquid chromatography (HPLC) analysis - see D17, page 316, last paragraph. The results of

D17 showed that soy-based foodstuffs or dietary supplements, such as soy-milk, soy flakes and textured soy, contain isoflavone phytoestrogens, and that these isoflavones were extracted and analyzed by HPLC in the context of their association with the incidence of human diseases. In paragraph [0030] of the opposed patent, it is stated that an alternative strategy for making available phyto-oestrogens in a purified form is to be considered.

Therefore, no inventive activity is involved in using an isoflavone-phytoestrogen extract of clover or soy for the preparation of the medicament above.

Therefore, the "invention" is obvious over D8 and the general common knowledge as stated in the opposed patent.

VI.2. Lack of inventive step of the dependent claims

Since claims 2 to 11 are dependent on claim 1, claims 2 to 11 also lack inventive step. Moreover, the dependent claims do not contain any technical features the incorporation of which into claim 1 would render claim 1 inventive.

VI.2.1. Claim 2 specifies that the medicament further comprises dietary suitable excipient. There is no inventive activity based on this feature as excipients suitable for dietary use are very well known in the art.

VI.2.2. Claim 3 specifies that the extract is from soya and claim 5 specifies that it is from clover. These limitations in the claims do not provide additional technical features over claim 1, and thus cannot establish an inventive step as it has been demonstrated with respect to claim 1.

VI.2.3. Claim 5 specifies that the extract is from soya hypocotyls. It has been known that hypocotyls have a high content of isoflavones (see page 7 of the application as filed). It

is obvious to use a specific part of a plant that has a high content of a desired compound as starting material for making an extract.

VI.2.4. Claims 6 to 10 specify the identities of the isoflavones, the amounts present in the unit dose, ratios of different isoflavones, and regimes of administration. Without a demonstrated benefit associated with these features, they have to be considered as mere selections without any associated technical effects. We note that the ratio of the isoflavones recited in claim 7 lies within the ratio of the isoflavones described in D10 for isolated isoflavones from red clover. We also note that the presence of lignans in soy-based diets and their association with benefits in the context of prostate cancer is discussed in D17. Like claim 1, no inventive step can be found for the subject matter of these dependent claims.

VI.2.5. Claim 11 specifies the unit dosage to be a tablet or capsule. There is no inventive activity in the selection of these features. Like claim 1, no inventive step can be recognised in claim 11.

VII. The Opposed Patent Does Not Disclose The Invention In A Manner Sufficiently Clear And Complete To Be Carried Out By A Person Skilled In The Art (Art. 100 (b) EPC and Art. 83 EPC)

VII.1. Preparation of the extracts

When looking at the description of the patent, example 1 describes a preparation of a red clover product according to the invention. Specifically in paragraph [0072], an extraction process is described and the final step of that process reads:

> *"The supernatant is separated from the undissolved plant material and the organic solvent removed by distillation. The aqueous supernatant then is concentrated, typically by distillation".*

This means the plant extract is in the aqueous phase and the aqueous phase is to be concentrated. There is no indication to what concentration the extract is to be concentrated, but it appears to be clear that the solvent is not necessarily completely removed, i.e. the final end product is not a dry powder but is probably a concentrated liquid.

Example 3 describes the administration of a red clover extract which is in the form of a dry powder of which the concentration of isoflavones is given. However, example 3 is not prepared according to the invention, and thus does not provide any information with respect to the invention.

It is the extract which is to be used as a medicament for administration to humans. Therefore, all indications in the claims and the description which specify an amount for administration (for example claim 8), do not provide any clear guidance to the person skilled in the art, since they refer to the amount of the extract but the concentration of isoflavones in that extract is undetermined and varies with the extent to which the solvent has been distilled off. Of course the concentration of isoflavones also depend on the solvent and extraction conditions used in the extraction step.

There is no information at all for the preparation of a soy extract. Example 2 only describes the separation of soy hypocotyls and example 4 describes the administration of a hypocotyl powder, i.e. milled hypocotyls, which is added to a diet.

VII.2. Treatment of symptoms or diseases

As mentioned already under III., the opposed patent lacks information on the treatment of prostate cancer and menopausal symptoms. Example 4 describes the administration of soy hypocotyl powder to a total of 15 people, 8 of which were women. The age of the women is not indicated, i.e., there is no indication whether the women are in the climacterium. The women are not prone to prostate cancer and the men are not subject to menopausal symptoms. There is no control group. The example reportedly show a significant fall of cholesterol levels for individuals having cholesterol levels greater than average.

From this example, no information can be derived with respect to the treatment of prostate cancer, menopausal symptoms or pre-menstrual symptoms. It is submitted that the only information given to the person skilled in the art with respect to administration and treatment is to reduce the level of cholesterol. We note that there is no explicit teaching of the concentration of the isoflavones to be administered for the treatment of the three conditions recited in claim 1. There is no teaching on how the extract is to be prepared, what the composition should contain. However, according to paragraph [0047] of the opposed patent, the concentration and actual isoflavones present in a composition appear to be important for not having any detrimental effect in humans. This fact is also supported by D6 wherein some detrimental effects of phyto-oestrogens in humans have been reported, and by Mr. Kelly's comment in his declaration that phyto-estrogens are generally noxious and may have a deleterious role in various diseases.

Given the lack of precise information, the skilled person is left to experiment in order to identify the efficacious concentration range and isoflavone composition for treating prostate cancer, pre-menstrual symptoms and menopausal symptoms. In particular with respect to treatment of humans, high safety standards apply. Given the lack of precise information in the opposed patent, identifying the appropriate range and composition must be considered as undue burden (T312/88).

We also point out that the Hughes declaration, submitted by the applicant during examination on October 21, 2003, was particularly critical of the reliability of the epidemiological data and the teachings presented in D8. However, the applicant relied on similar epidemiological data to support the alleged invention (see priority document, page 6, lines 3 to 15 and; paragraphs [0020 to [0038] of the opposed patent). More importantly, the applicant neglected to provide any data to enable the alleged invention in the treatment of prostate cancer, and menopausal symptoms. Thus, if the applicant maintains that the alleged invention contains an inventive step because the prior art teachings are considered to be too speculative, the disclosure of the opposed patent would likewise fails to provide sufficient details and certainty to allow one of skill in the art to carry out the alleged invention.

VIII. Conclusion

In our view we have been able to demonstrate that none of the granted claims is patentable according to the requirements of Art. 54, 56, 83 and 123 EPC. The patent is to be revoked in its entirety.

Dr. Mathias Ricker
European Patent Attorney
Association Number 215

Enclosures
Duplicate of this letter
Form 1010 in the amount of € 610,-
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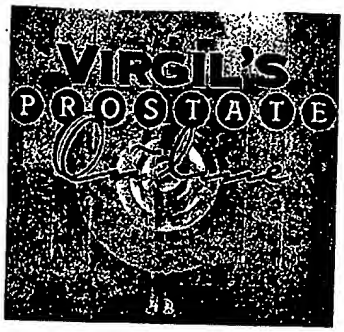

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Until There is a Cure, We Will Provide the Means to Cope!!

August, 1998

Selenium and "Man's Best Friend": Warriors Against Prostate Cancer

As the incidence of prostate cancer increases, so do the number of homeopathic and complementary treatments which promise a "cure" for the disease or its side-effects. many are pure promotional hype, unsubstantiated science or anecdotal claims without supporting data all geared to separate you from your money in return for a glimmer of hope.

Yet we know that certain vitamins and minerals, herbs and foods CAN have a positive therapeutic effect. This month we will look at one of them -- **Selenium**.

Dr. David Waters, Associate Professor of Surgery and Comparative Oncology, in the School of Veterinary Medicine at Purdue University has done significant groundbreaking research on the positive impact of selenium in prostate cancer chemoprevention utilizing several canine models.

Dr. Waters, please solve the confusion for me and I'm sure our viewers, how does veterinary medicine and the use of dogs relate to human prostate cancer?

DW: The dog is the only non-human creature in which prostate cancer naturally occurs with a high degree of frequency. Overall, cancer is the primary cause of death in 40% of dogs. In our work we have seen dogs manifest many of the same cancers as humans, i.e. breast, lymphoma, melanoma, prostate, etc.; thus we concluded that

a critical hypothesis could be developed that would attempt to identify prostate cancer markers in dogs that would have relevance for chemoprevention potential for humans.

VS: How do dogs develop prostate cancer and are there tests, like the PSA blood screen, which provide a marker for canines and possibly humans?

DW: Just as with humans, we don't know why dogs develop prostate cancer and the PSA is not a valid test because the production of PSA is restricted to humans and non-human primates. Again, as with humans, there has not been a lot of attention paid to prostate cancer in dogs. We have begun the first systematic study of the disease because our preliminary research has shown that 55% of the dogs autopsied at time of death had prostatic intraepithelial neoplasia (PIN), which is the putative pre-cursor of many human prostate cancers, even though there had been no clinical signs of prostatic disease.

VS: Is this marker - PIN - significant?

DW: Definitely, because it tells us that early carcinogenic events may be occurring within the dog's prostate, but the animals aren't living long enough for the cancer to be identified or treated as such. If we can identify the elements associated with this marker we can conceivably develop a chemopreventive strategy viable for the treatment of prostate cancer in humans. In addition to PIN, we will be looking for correlation between other bio-markers, such as the level of selenium in dog toenails and the presence 8OHdG in the urine with the incidence of prostate cancer.

VS: You've recently been funded by the Department of Defense for a research proposal on the prevention of prostate cancer. Tell us about it.

DW: We are looking to validate the dog as a

pre-clinical model for the testing of chemopreventive agents for prostate cancer. We are using dogs because, compared with rodents, they will present results more directly translatable to humans. We envision a short term study lasting approximately 2 years compared with 8 or more years using conventional protocols in human trials because the pet dog, in its diet and environment and breeding, more closely approximates the conditions under which human males will present prostate cancer. Dogs live in homes and locales as humans, approximately 30% of their diet comes from table scraps, they are not as in-bred as rodents and the ability to monitor their diet, etc. is easier to manage than with humans.

VS: Selenium, as a mineral supplement, has traditionally been used as an aid to preventing heart disease as well as an antioxidant enzyme. What is the benefit relative to prostate cancer?

DW: Our interest in Selenium and prostate cancer chemoprevention is based on our work which we did with Dr. David Bostwick(at the Mayo Clinic) and the data from Dr. Larry Clark (University of Arizona) that Selenium lowered the overall incidence of certain cancers, including prostate. It seems logical that we should determine the effect of selenium supplements on prostate carcinoma in a controlled investigation. We believe that selenium can have antioxidant properties which will reduce cumulative oxidant damage within the prostate. Significant benefits could be achieved by preventing or slowing the growth of the disease, since aging is the most significant risk factor.

VS: In your initial research, at what dosage levels will you evaluate for chemopreventive results?

DW: Our initial dosage in the dog models will be 3mcg and 6mcg per kilo of body weight which translates roughly into the daily

recommendations of 200 mcg or 400 mcg in the human studies underway.

VS: At this point has there been enough evidence to recommend the usage of selenium in humans as a therapy against prostate cancer, or as a preventive measure against contraction of the disease?

DW: Our studies are only in the preliminary stage. We will receive funding from the Department of Defense in October '98 at which time we will begin to implement our research protocols. We are at least 2 years away from being able to definitely state that it is an effective chemopreventive agent.

VS: We have noted that some other "natural" substances such as, Vitamins C, D, and E and soy, calcium, magnesium and zinc are prostate cancer fighters. Can you comment on how Selenium interacts with these or other substances either positively or negatively?

DW: As part of our study we will also be testing DHEA. There is some concern that it may actually aggravate prostate cancer because of its pro-androgenic properties. But this is the strength of our experimental design; we will be running a double-blind trial using a placebo and controlled selenium dosages. We will be able to determine the efficacy of selenium in preventing/controlling the growth of pre-malignant lesions and prostate cancer in our subjects. Compare this with humans in a clinical trial who are being evaluated for a particular therapy, yet may be "contaminating" the results based on their taking some other self-medicated substances, thereby potentially altering or invalidating the data. Ultimately we anticipate that selenium may be used in combination with some other chemopreventive agent in a "cocktail" supplement.

VS: As with most clinical trials I would assume that you need subjects to help validate your

hypotheses. What kind of canine volunteers are you looking for?

DW: We are looking for 360 healthy, sexually intact adult male dogs of various breeds. We have constructed an algorithm which factors in age and body size to produce an expected life span which would equate to a human life span. The dogs that will be in the study would equate to a human age range of 50 to 70 years. Although we would expect to pull most of our subjects from the Chicago Metro/Indiana area, we are seeking to create collaborations with veterinarians and pet owners around the country so that we can more effectively accrue cases.

VS: Will the dogs be exposed to any prostate cancer cells/tissue, viruses or any other potentially toxic agents?

DW: Absolutely not!!! They will receive either a sugar pill or selenium capsule daily for the rest of their lives, which we would estimate would be in the 2 year cycle of our study. They will also receive regular checkups to monitor their diet and overall health.

VS: After the dogs die of whatever natural causes, what will your procedure be?

DW: We will conduct an autopsy and evaluate the **total** prostate which will yield more data about the disease versus just a biopsy. We will also measure 8OHdG and toenail selenium levels to test our hypothesis in identifying markers viable for human prostate cancer.

VS: If our viewers wish to get more information on the study, how can they contact you?

DW: They should contact me via e-mail at: = **waters@vet.purdue.edu**

VS: How can our viewers volunteer their dogs to be = participants in this trial? What is the criteria for acceptance and are there any stipends

provided for participation?

DW: Dog owners can contact me directly or, better yet, through their local veterinarians as soon as possible and definitely prior to October 1st. The algorithmic-adjusted age of the dog will be the primary factor assuming that they meet the other criteria of health and sexual condition.

We will not be offering any stipend because of the limits of our study funding. However, prostate cancer touches the lives of over 200,000 men annually and a multiple number of family members and friends. There are millions of people who have seen the devastating impact of prostate cancer on their lives. We would sincerely hope that they would see this as a chance to stem the carnage of this disease on any other family by volunteering their animals to be a part of this effort. We only need **360** dogs out of the entire U.S. population!!!!

Thank you again, Dr. Waters, for sharing this exciting information and also providing us with some tangible evidence of the Department of Defense's Prostate Cancer Research support initiatives.

NOTE: We strongly urge our viewers to contact their Congressmen and Senators to push for more Federal dollars for prostate cancer research.

For additional information on selenium and prostate cancer, we recommend that you review Dr. Larry Clark's study. **Click Here** to see the article.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5: A61K 31/35 — A1

(11) International Publication Number: WO 94/23716

(43) International Publication Date: 27 October 1994 (27.10.94)

(21) International Application Number: PCT/US94/04189

(22) International Filing Date: 15 April 1994 (15.04.94)

(30) Priority Data: 08/049,006 — 16 April 1993 (16.04.93) — US

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

(57) Abstract

A method is provided for preventing or treating symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of an isoflavonoid. The invention also features a therapeutic dietary product, containing isoflavonoids, for preventing or treating symptoms of conditions resulting from reduced or altered levels of endogenous estrogen.

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METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

Background of the Invention

The present invention relates to therapies for the prevention and treatment of menopausal and premenstrual symptoms.

It has long been recognized that the sharp reduction in endogenous estrogen levels which occurs prior to menopause causes a variety of unpleasant symptoms, e.g., hot flashes, nausea, nervousness, and malaise. Currently, the symptoms of menopause are treated by estrogen replacement therapy, which has recently been shown to increase the risk of certain types of cancer, such as endometrial cancer and breast cancer. Changes in levels of endogenous estrogen may also be responsible for "premenstrual syndrome", a condition occuring in younger women prior to menstruation. Premenstrual symptoms are treated with a variety of hormonal and nonhormonal therapies, which may cause side effects. Safer and more effective therapies for both conditions continue to be sought.

Summary of the Invention

The inventors have found that isoflavonoids, which are constituents of soy beans and other plants, effectively reduce the symptoms of conditions which are caused by reduced or altered levels of endogenous estrogen, e.g., menopause, and premenstrual syndrome. Without being bound by any theory, it is believed that the isoflavonoids bind to estrogen receptors, and thus exert an estrogenic response. These compounds, which are present naturally in soy-based and other plant-based foods, are safe and cause no significant side-effects.

Isoflavonoids which may be administered according to the invention include genistein, daidzein, Biochanin A,

formononetin, O-desmethylangolensin, and equol; these may be administered alone or in combination.

Accordingly, in one aspect, the invention features a method of preventing or treating the symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of at least one isoflavonoid. The isoflavonoid may be administered in any suitable form, e.g., in the form of a plant extract rich in isoflavonoids or in the form of a purified or synthesized isoflavonoid.

In another aspect, the invention features a therapeutic dietary product for preventing or treating symptoms resulting from reduced or altered levels of endogenous estrogen. The dietary product preferably includes a soy extract containing enriched isoflavonoids, provided in a palatable food carrier, e.g., a confectionary bar, biscuit, cereal or beverage.

Other features and advantages of the invention will be apparent from the Description of the Preferred Embodiments thereof, and from the claims.

Description of the Preferred Embodiments

Isoflavonoids are naturally occurring substances, found primarily in soy beans. These compounds are also found in lower concentrations in many other plants. Isoflavonoids can thus be administered to a patient by placing the patient on a diet containing high levels of soy-based food products, e.g., tofu, miso, soybeans, aburage, atuage and koridofu, or other plant products rich in isoflavonoids.

These products may not be readily available in all geographic regions (most of these foods are served predominantly in Japan), and are not be palatable to many women, particularly those accustomed to Western-style food.

Accordingly, an isoflavonoid-containing fraction can be extracted from a soy or plant product. It is preferred that the isoflavonoids be extracted and concentrated from soy bean or soy powder. Isoflavonoids are also available commercially in substantially pure form. The concentrated isoflavonoid is preferably included in a food carrier to form a dietary product. Any type of palatable carrier may be used, but, as the isoflavonoid concentrate has a strong flavor, it is preferred that the carrier include suitable flavorings to impart a different, more palatable flavor. The dietary product may be any type of food product, e.g., a confectionary bar, biscuit, cereal or beverage.

It is preferred that the dietary product contain at least 30 mg/serving total isoflavonoids. The isoflavonoid concentrate included in the dietary product preferably includes a blend primarily comprised of genistein and daidzein. The concentrate typically also contains lower levels of other isoflavonoids. Most preferably, the dietary product contains from about 10 to 30 mg/serving, more preferably about 20 mg/serving of genistein, and from about 5 to 10 mg/serving, more preferably about 7 mg/serving of daidzein. Preferably, a dietary product containing the preferred dosage of isoflavonoids would be consumed at least once per day, preferably 1 to 2 times per day depending upon the severity of the woman's symptoms.

While it is preferred that the isoflavonoid be administered in the form of a dietary product, if desired the isoflavonoid could be administered, preferably in similar dosages, in medicament form, e.g., mixed with a pharmaceutically acceptable carrier to form a tablet, powder or syrup.

Example

The connection between diet and estrogen excretion was studied in Japanese women and men, and in a few children. The women's mean age was 50.4 (SD 18.0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. Isoflavonoid excretion in the urine was measured in a group of three men, three women, and three children living in Kyoto and consuming the traditional diet. We found a very high excretion of isoflavonoids in the urine of these subjects. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15.5 umol per 24h in a man) and two other isoflavonoids, daidzein and equol (Table 1). All these compounds bind to estrogen receptors and have weak estrogenic activity. The excretion of the isoflavonoids in urine of the Japanese women was much higher than previously determined levels in American and Finnish women (Table 1). Excretion was high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than the levels of endogenous estrogens excreted by normal omnivorous women consuming a western or oriental diet (Table 1).

The excretion of the isoflavonoids in urine was associated with intake of soy products such as tofu, miso, aburage, atuage, koridofu, soybeans, and boiled beans.

It is known that Japanese women have a lower incidence of menopausal symptoms and premenstrual symptoms than the American and Finnish women.

Table 1

Urinary isoflavonoid or estrogen (nmol/day)	Japanese/ Oriental	American	Finnish
Genistein	3440(n=3)	. .	32.1(n=12)
Daidzein	2600(n=10)	216(n=21)	40.5(n=12)
Equol	2600(n=10)	62.8(n=21)	44.2(n=12)
Oestrone (postmenstrual)	4.48(n=9)	. .	4.48(n=10)
Oestradiol (postmenstrual)	0.76(n=9)	. .	0.94(n=10)
Oestriol (postmenstrual)	4.48(n=9)	. .	4.44(n=10)

CLAIMS

1. Use of an isoflavonoid in the preparation of a medicament for preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen.

2. The use of claim 1, wherein said isoflavonoid is selected from the group consisting of genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin and equol.

3. The use of claim 1 wherein said isoflavonoid is in a unit dosage of at least 30 mg.

4. The use of claim 1 wherein genistein and daidzein isoflavonoids are present in said medicament.

5. The use of claim 4 wherein said isoflavonoid comprises from about 10 to 30 mg genistein and from about 5 to 10 mg daidzein.

6. The use of claim 1 wherein said medicament is in the form of a dietary product.

7. The use of claim 6 wherein said dietary product contains at least 30 mg/serving of said isoflavonoid.

8. The use of claim 6 wherein said dietary product is a confectionery bar containing said isoflavonoid.

9. The use of claim 6 wherein said dietary product is a cereal containing said isoflavonoid.

10. The method of claim 6 wherein said dietary product is a biscuit containing said isoflavonoid.

11. The method of claim 6 wherein said dietary product is a beverage containing said isoflavonoid.

12. A dietary product for preventing or treating symptoms of menopause, premenstrual syndrome, or conditions resulting from reduced or altered levels of endogenous estrogen, comprising at least one isoflavonoid provided in a non-soy-based palatable food carrier.

13. The dietary product of claim 12 comprising genistein and daidzein isoflavonoids.

14. The dietary product of claim 12 wherein the food carrier is a confectionery bar.

15. The dietary product of claim 12 wherein the food carrier is a cereal.

16. The dietary product of claim 12 wherein the food carrier is a biscuit.

17. The dietary product of claim 12 wherein the food carrier is a beverage.

18. The dietary product of claim 12 wherein the food carrier contains an amount of the isoflavonoid which is effective in reducing the symptoms.

19. The dietary product of claim 18 comprising at least about 30 mg isoflavonoids per serving.

20. The dietary product of claim 13 wherein said dietary product comprises from about 10 to 30 mg/serving genistein and from about 5 to 10 mg/serving daidzein.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04188

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 31/35-
US CL :514/456, 899

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/456, 899

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS AND CAS ONLINE: ISOFLAVIN?, PMS, ESTRO?, PREMENSTRUAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 3,864,362 (FEUER ET AL.) 04 FEBRUARY 1975, COLUMN 1, LINE 33 - COLUMN 2, LINE 44.	1-20 ------ 1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 MAY 1994	Date of mailing of the international search report JUL 20 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KIMBERLY JORDAN Telephone No. (703) 308-1235

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Most recent event

Application deemed to be withdrawn
Date of publication 07-02-2001 [2001/06]

Publication numbers, publication type and publication dates

EP0693927 A1 27-10-1994 [1996/05]
WO9423716 27-10-1994 [1996/05]

Application numbers and filing date

EP19940914194 (94914194.9)
Date of filing 15-04-1994 [1996/05]
WO1994US04189

Date of publication of search report

Date of supplementary search report 11-04-1997 [1997/22]
Date of international search report 27-10-1994
International Searching Authority US

Priority number, priority date

US19930049006 16-04-1993 [1996/05]

Classification (IPC) and bulletin number

A61K31/35, A61K31/12 [1997/22]
[
A61K31/35 [1996/05]
]

Designated states

AT , BE , CH , DE , DK , ES , FR , GB , GR , IE , IT , LI , LU , NL , PT , SE [1996/05]

English title

METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS [1996/05]

French title

PROCEDE DE TRAITEMENT DES SYMPTOMES MENAUPOSIQUES ET PREMENSTRUELS [1996/05]

German title

VERFAHREN ZUR BEHANDLUNG MENOPAUSALER UND PRÄMENTRUELLER SYMPTOME [1996/05]

Designated states, applicant name, address

FOR ALL DESIGNATED STATES
TUFTS UNIVERSITY SCHOOL OF MEDICINE
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03 / ADLERCREUTZ, Herman, Departm.of Clinical Chemistry /

University of Helsinki, Meilahti Hospital / FIN-000290 Helsinki / FI [1996/05]

- **Representative name, address**

Allman, Peter John, et al
MARKS & CLERK, Sussex House, 83-85 Mosley Street
Manchester M2 3LG/GB [1996/05]

Filing language

EN

Procedure language

EN

Location of file and fax number for file inspection requests

Application is treated in (/fax-nr)	MUNICH/(+49-89) 23994465

PCT: Acts performed for entry into EPO regional phase

Acts performed for entry into the regional phase	11-10-1995
- National basic fee paid	11-10-1995
- Search fee paid	11-10-1995
- Designation fee(s) paid	11-10-1995
- Examination fee paid	11-10-1995

Examination procedure

Date of request for preliminary examination	09-11-1994
request for examination	11-10-1995 [1996/05]
Examination report(s) date dispatch/time-limit/reply	A.96(2), R.51(2) 16-02-2000/M06/00000000

Application withdrawn or deemed to be withdrawn

Communication, that the application is deemed to be withdrawn

date dispatch/legal effect date	21-09-2000/28-08-2000 [2001/06]
Reason	A.96(3)

Renewal fees

Renewal fee A.86 (patent year / paid)	03/25-03-1996 04/24-03-1997 05/26-03-1998 06/23-04-1999 07/03-04-2000

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[XP] AM. J. CLIN. NUTR., vol. 60, no. 3, 1994, pages 333-340, XP000647466 CASSIDY ET AL.: "Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women."
[ ] See also references of WO 9423716A1
[End of Data]

Return to Search Screen
10-06-2005 16:12:03

PCT/US 94/04189

6

BAR CODE LABEL

U.S. PATENT APPLICATION

08/249,194.9

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT
08/049,006	04/16/93	514	1205

APPLICANT

SHERWOOD L. GORBACH, CHESTNUT HILL, MA; BARRY R. GOLDIN, WEST NEWTON, MA; HERMAN ADLERCREUTZ, HELSINKI, FINLAND.

CONTINUING DATA********
VERIFIED

REC'D 31 MAY 1994
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FOREIGN/PCT APPLICATIONS********
VERIFIED

PRIORITY DOCUMENT

FOREIGN FILING LICENSE GRANTED 08/19/93 ***** SMALL ENTITY *****

STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS	FILING FEE RECEIVED	ATTORNEY DOCKET NO
MA	0	22	2	$442.00	05495/203001

ADDRESS

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225 FRANKLIN STREET
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TITLE

METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application which is identified above.

By authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

Date APR 25 1994 Certifying Officer

31

PATENT APPLICATION SERIAL NO. 08/049006

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

080 AT 05/03/93 08049006 1 201 377.00 CK

PTO-1556
(5/87)

APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

APPLICANT: SHERWOOD L. GORBACH, BARRY R. GOLDIN AND HERMANN ADELCREUTZ

"Express Mail" mailing label number [illegible]

Date of Deposit April 16, 1993

I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington DC 20231

[illegible]

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377.00 201 #

09/049006

ATTORNEY DOCKET NO: 05495/003001

METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

Background of the Invention

The present invention relates to therapies for the prevention and treatment of menopausal and premenstrual symptoms.

It has long been recognized that the sharp reduction in endogenous estrogen levels which occurs prior to menopause causes a variety of unpleasant symptoms, e.g., hot flashes, nausea, nervousness, and malaise. Currently, the symptoms of menopause are treated by estrogen replacement therapy, which has recently been shown to increase the risk of certain types of cancer, such as endometrial cancer and breast cancer. Changes in levels of endogenous estrogen may also be responsible for "premenstrual syndrome", a condition occuring in younger women prior to menstruation. Premenstrual symptoms are treated with a variety of hormonal and nonhormonal therapies, which may cause side effects. Safer and more effective therapies for both conditions continue to be sought.

Summary of the Invention

The inventors have found that isoflavonoids, which are constituents of soy beans and other plants, effectively reduce the symptoms of conditions which are caused by reduced or altered levels of endogenous estrogen, e.g., menopause, and premenstrual syndrome. Without being bound by any theory, it is believed that the isoflavonoids bind to estrogen receptors, and thus exert an estrogenic response. These compounds, which are present naturally in soy-based and other plant-based foods, are safe and cause no significant side-effects. Isoflavonoids which may be administered according to the invention include genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin, and equol; these may be administered alone or in combination.

Accordingly, in one aspect, the invention features a method of preventing or treating the symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of at least one isoflavonoid. The isoflavonoid may be administered in any suitable form, e.g., in the form of a plant extract rich in isoflavonoids or in the form of a purified or synthesized isoflavonoid.

In another aspect, the invention features a therapeutic dietary product for preventing or treating symptoms resulting from reduced or altered levels of endogenous estrogen. The dietary product preferably includes a soy extract containing enriched isoflavonoids, provided in a palatable food carrier, e.g., a confectionary bar, biscuit, cereal or beverage.

Other features and advantages of the invention will be apparent from the Description of the Preferred Embodiments thereof, and from the claims.

Description of the Preferred Embodiments

Isoflavonoids are naturally occurring substances, found primarily in soy beans. These compounds are also found in lower concentrations in many other plants. Isoflavonoids can thus be administered to a patient by placing the patient on a diet containing high levels of soy-based food products, e.g., tofu, miso, soybeans, aburage, atuage and koridofu, or other plant products rich in isoflavonoids.

These products may not be readily available in all geographic regions (most of these foods are served predominantly in Japan), and are not be palatable to many women, particularly those accustomed to Western-style food.

Accordingly, an isoflavonoid-containing fraction can be extracted from a soy or plant product. It is preferred

that the isoflavonoids be extracted and concentrated from soy bean or soy powder. Isoflavonoids are also available commercially in substantially pure form. The concentrated isoflavonoid is preferably included in a food carrier to form a dietary product. Any type of palatable carrier may be used, but, as the isoflavonoid concentrate has a strong flavor, it is preferred that the carrier include suitable flavorings to impart a different, more palatable flavor. The dietary product may be any type of food product, e.g., a confectionary bar, biscuit, cereal or beverage.

It is preferred that the dietary product contain at least 30 mg/serving total isoflavonoids. The isoflavonoid concentrate included in the dietary product preferably includes a blend primarily comprised of genistein and daidzein. The concentrate typically also contains lower levels of other isoflavonoids. Most preferably, the dietary product contains from about 10 to 30 mg/serving, more preferably about 20 mg/serving of genistein, and from about 5 to 10 mg/serving, more preferably about 7 mg/serving of daidzein. Preferably, a dietary product containing the preferred dosage of isoflavonoids would be consumed at least once per day, preferably 1 to 2 times per day depending upon the severity of the woman's symptoms.

While it is preferred that the isoflavonoid be administered in the form of a dietary product, if desired the isoflavonoid could be administered, preferably in similar dosages, in medicament form, e.g., mixed with a pharmaceutically acceptable carrier to form a tablet, powder or syrup.

Example

The connection between diet and estrogen excretion was studied in Japanese women and men, and in a few

children. The women's mean age was 50.4 (SD 18.0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. Isoflavonoid excretion in the urine was measured in a group of three men, three women, and three children living in Kyoto and consuming the traditional diet. We found a very high excretion of isoflavonoids in the urine of these subjects. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15.5 umol per 24h in a man) and two other isoflavonoids, daidzein and equol (Table 1). All these compounds bind to estrogen receptors and have weak estrogenic activity. The excretion of the isoflavonoids in urine of the Japanese women was much higher than previously determined levels in American and Finnish women (Table 1). Excretion was high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than the levels of endogenous estrogens excreted by normal omnivorous women consuming a western or oriental diet (Table 1).

The excretion of the isoflavonoids in urine was associated with intake of soy products such as tofu, miso, aburage, atuage, koridofu, soybeans, and boiled beans.

It is known that Japanese women have a lower incidence of menopausal symptoms and premenstrual symptoms than the American and Finnish women.

Other embodiments are within the claims.

Table 1

Urinary isoflavonoid or estrogen (nmol/day)	Japanese/ Oriental	American	Finnish
Genistein	3440(n=3)	. .	32.1(n=12)
Daidzein	2600(n=10)	216(n=21)	40.5(n=12)
Equol	2600(n=10)	62.8(n=21)	44.2(n=12)
Oestrone (postmenstrual)	4.48(n=9)	. .	4.48(n=10)
Oestradiol (postmenstrual)	0.76(n=9)	. .	0.94(n=10)
Oestriol (postmenstrual)	4.48(n=9)	. .	4.44(n=10)

CLAIMS

1. A method of preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen, said method comprising administering to the woman an effective amount of an isoflavonoid.

2. The method of claim 1, wherein said isoflavonoid is selected from the group consisting of genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin and equol.

3. The method of claim 1 wherein said isoflavonoid is administered in a dosage of at least 30 mg.

4. The method of claim 3 wherein said isoflavonoid is administered in said dosage at least once per day.

5. The method of claim 1 wherein genistein and daidzein isoflavonoids are coadministered.

6. The method of claim 5 wherein said isoflavonoid comprises from about 10 to 30 mg genistein and from about 5 to 10 mg daidzein.

7. The method of claim 1 wherein said isoflavonoid is administered in the form of a dietary product.

8. The method of claim 7 wherein said dietary product contains at least 30 mg/serving of said isoflavonoid.

9. The method of claim 7 wherein said dietary product is a confectionery bar containing said isoflavonoid.

10. The method of claim 7 wherein said dietary product is a cereal containing said isoflavonoid.

11. The method of claim 7 wherein said dietary product is a biscuit containing said isoflavonoid.

12 The method of claim 7 wherein said dietary product is a beverage containing said isoflavonoid.

13. The method of claim 7 wherein said dietary product is consumed by said woman at least once per day.

14. A dietary product for preventing or treating symptoms of menopause, premenstrual syndrome, or conditions resulting from reduced or altered levels of endogenous estrogen, comprising at least one isoflavonoid provided in a non-soy-based palatable food carrier.

15. The dietary product of claim 14 comprising genistein and daidzein isoflavonoids.

16. The dietary product of claim 14 wherein the food carrier is a confectionery bar.

17. The dietary product of claim 14 wherein the food carrier is a cereal.

18. The dietary product of claim 14 wherein the food carrier is a biscuit.

19. The dietary product of claim 14 wherein the food carrier is a beverage.

20. The dietary product of claim 14 wherein the food carrier contains an amount of the isoflavonoid which is effective in reducing the symptoms.

21. The dietary product of claim 20 comprising at least about 30 mg isoflavonoids per serving.

22. The dietary product of claim 15 wherein said dietary product comprises from about 10 to 30 mg/serving genistein and from about 5 to 10 mg/serving daidzein.

METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

Abstract of the Disclosure

A method is provided for preventing or treating symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of an isoflavonoid. The invention also features a therapeutic dietary product, containing isoflavonoids, for preventing or treating symptoms of conditions resulting from reduced or altered levels of endogenous estrogen.

30222

PATENT
ATTORNEY DOCKET NO: 05495/003001

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS, the specification of which

- ☒ was filed on APRIL 16, 1993 as Application Serial No 08/049,006 and was amended on _____.
- ☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: William E. Booth, Reg No 28,933; Barry E. Bretschneider, Reg. No. 28,055; Paul T. Clark, Reg. No. 30,162; Willis M. Ertman, Reg No. 18,658; David L. Feigenbaum, Reg. No. 30,378; John W. Freeman, Reg. No. 29,066; Timothy A. French, Reg. No. 30,175; Alan H. Gordon, Reg No. 26,168; Gilbert H. Hennessey, Reg. No. 25,759; Charles Hieken, Reg. No. 18,411; Robert E. Hillman, Reg. No. 22,837; G. Roger Lee, Reg. No. 28,963; Steven E. Lipman, Reg No. 30,011; Gregory A. Madera, Reg. No. 28,878; Ralph A. Mittelberger, Reg No 33,195; Ronald E. Myrick, Reg No. 26,315; Frank P. Porcelli, Reg. No. 27,374; Eric L. Prahl, Reg. No 32,590; Alan D. Rosenthal, Reg. No. 27,833; John M. Skenyon, Reg. No. 27,468; Michael O. Sutton, Reg No. 26,675; Rene D. Tegtmeyer, Reg. No 33,567; John N. Williams, Reg. No. 18,948; Gary A. Walpert, Reg. No. 26,098; Charles C. Winchester, Reg No. 21,040; and Celia H. Ketley, Reg. No. 33,524.

Address all telephone calls to Paul T. Clark at telephone number 617/542-5070.

Address all correspondence to Paul T. Clark, Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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Inventor's Signature: _____ Date: 6/25/93

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Citizen of: U.S.

Post Office Address: Same

COMBINED DECLARATION AND POWER OF ATTORNEY CONTINUED

2. (a) Full Name of Inventor: Barry R. Goldin

Inventor's Signature: [signature] Date: [illegible]

Residence Address: 38 Adella Avenue, West Newton, Massachusetts 02165 MA

Citizen of: U.S.

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Inventor's Signature: [signature] Date: [illegible]

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Citizen of: Finland

Post Office Address: Same as above

ATTORNEY DOCKET NO. 05495

Applicant or Patentee: SHERWOOD L. GORBACH ET AL.
Serial or Patent No.: 08/049,006
Filed or Issued: APRIL 16, 1993
For: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: TUFTS UNIVERSITY SCHOOL OF MEDICINE
Address of Organization: BOSTON, MA
Type of Organization: UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

[X] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE:)
(CITATION OF STATUTE:)
[ ] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE:)
(CITATION OF STATUTE:)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1. for purposes paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the inv ion entitled METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS by inventor(s) SHERWOOD L. GORBACH, BARRY R. GOLDIN, and HERMAN ADLERCREUTZ described in

[ ] the specification filed herewith.
[X] application serial no. 08/049,006, filed APRIL 16, 1993.
[ ] patent no. , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name: _____

Address: _____

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name: ROBERT GARRISON

Title: DIRECTOR, PATENTS & LICENSING

Address: TUFTS UNIVERSITY, PACKARD HALL, MEDFORD, MA 02155

Signature: _____ Date: _____

D3

(19) JAPANESE PATENT OFFICE

PATENT ABSTRACTS OF JAPAN

(11) Publication number: 61246124 A

(43) Date of publication of application: 01.11.86

(51) Int. Cl A61K 31/35
// C07D311/30

(21) Application number: 60089770

(22) Date of filing: 24.04.85

(71) Applicant: YAMANOUCHI PHARMACEUT CO LTD OGAWARA HIROSHI

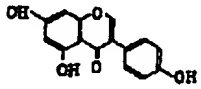
(72) Inventor: OGAWARA HIROSHI
WATANABE SHUNICHI

(54) CARCINOSTATIC AGENT

(57) Abstract:

PURPOSE: To provide a carcinostatic agent containing 5,7,4'-trihydroxyisoflavone as an active component and having tumor cell proliferation inhibiting activity and DNA-synthesis inhibiting activity.

CONSTITUTION: The objective agent contains 5,7,4'-trihydroxyisoflavone (general name: genistein) as an active component. Genistein is a compound separated from a certain kind of clover (Trifolium subterraneum L.) and is known to have week estrogen activity. It has been found newly that the compound is effective to inhibit the proliferation of tumor cell, the synthesis of DNA and the activity of tyrosine-specific phosphorylase. Coupled with the low acute toxicity, the compound is useful as a carcinostatic agent for the remedy of human and animal cancer, the remedy for diseases caused by the metastasis of cancer and the prevention of relapse of cancer. It is applied at a rate of usually 200W1,000mg daily in 1W4 divided doses.



D4

(19) JAPANESE PATENT OFFICE

PATENT ABSTRACTS OF JAPAN

(11) Publication number: 01258669 A
(43) Date of publication of application: 16.10.1989

(51) Int. Cl C07D311/40
C07D311/36, C07H 17/07

(21) Application number: 63083185
(22) Date of filing: 06.04.1988

(71) Applicant: KIKKOMAN CORP
(72) Inventor: OBATA AKIO
MATSUURA MASARU
HASHIMOTO HIKOTAKA

(54) PRODUCTION OF ISOFLAVON COMPOUND

(57) Abstract:

PURPOSE: To inexpensively obtain a large amount of aglycones from an extracted solution or ground substance of soybeans, by heating soybeans or ground soybeans at a specific temperature in immersion, grinding and/or enzyme reaction process to maximize β-glucosidase activity in soybeans.

CONSTITUTION: In producing an isoflavon compound having estrogen action, antioxidation action, antihemolytic action, antilipemic action, cholesterol-lowering action and carcinostatic action from an extracted solution of soybeans or a ground material thereof, soybeans or the ground material is heated to 45-55°C in one process of immersion process, grinding process and enzyme reaction process after grinding to maximize β-glucosidase in the soybeans to give an isoflavon compound containing a large amount of aglycones such as daizein or genistein which is a main substance of medicinal effects such as carcinostatic action among isoflavon compounds and has extremely high utility.

Meyer-Nachschlagewerke aus dem Bibliographischen Institut

Meyers Enzyklopädisches Lexikon
in 25 Bänden

Meyers Großes Universallexikon
in 15 Bänden

Meyers Neues Lexikon
in 8 Bänden

Meyers Großes Standardlexikon
in 3 Bänden

Meyers Großes Taschenlexikon
in 24 Bänden

Meyers Taschenlexikon
in 10 Bänden

Meyers Großes Handlexikon in Farbe

Meyers Taschenlexikon Biologie
in 3 Bänden

Meyers Taschenlexikon Geschichte
in 6 Bänden

Meyers Taschenlexikon Musik
in 3 Bänden

Meyers Handbücher
der großen Wissensgebiete

Meyers Enzyklopädie der Erde
in 8 Bänden

Die Erde: Meyers Großkarten-Edition

Meyers Großer Weltatlas

Meyers Neuer Handatlas

Meyers Neuer Atlas der Welt

Wie funktioniert das?

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Wie funktioniert das?

Der Mensch und seine Krankheiten

Herausgegeben und bearbeitet
von der Redaktion
Naturwissenschaft und Medizin
des Bibliographischen Instituts
unter der Leitung von
Karl-Heinz Ahlheim

Dritte, vollständig überarbeitete Auflage

Bibliographisches Institut Mannheim/Wien/Zürich
Meyers Lexikonverlag

249 zweifarbige Schautafeln,
8 mehrfarbige Schautafeln,
333 Textseiten,
zahlreiche Tabellen,
13 Registerseiten

CIP-Kurztitelaufnahme der Deutschen Bibliothek

Wie funktioniert das? Der Mensch und seine Krankheiten/
hrsg. u. bearb. von d. Red. Naturwiss. u. Medizin d. Bibliograph. Inst. unter d. Leitung von Karl-Heinz Ahlheim. – 3., vollst. überarb. Aufl. – Mannheim; Wien; Zürich: Bibliographisches Institut, 1984.
ISBN 3-411-02376-7
NE: Ahlheim, Karl-Heinz [Hrsg.]

Satz: Bibliographisches Institut und
Zechnersche Buchdruckerei, Speyer (Mono-Photo-System 600)
Druck: Zechnersche Buchdruckerei, Speyer
Bindearbeit: Klambt-Druck GmbH, Speyer
Printed in Germany
ISBN 3-411-02376-7

Vorwort zur dritten Auflage

Das über Jahre hin unverändert große Interesse an „Wie funktioniert das? – Der Mensch und seine Krankheiten" liegt sicher nicht zuletzt darin begründet, daß in diesem Buch die zahlreichen Krankheiten, mit denen wir uns im Laufe unseres Lebens herumplagen müssen, auf dem Hintergrund des normalen physiologischen Geschehens im Organismus geschildert werden: Funktion und Dysfunktion als integrale Ganzheit. Dieses bewährte Prinzip und die benutzerfreundliche synoptische Gegenüberstellung von Textseiten und die Textinformation ergänzenden und illustrierenden zweifarbigen Bildtafeln haben wir auch in der vorliegenden dritten Auflage des Buches beibehalten. Beibehalten wurde auch die thematische Gliederung nach Funktionskreisen. Erhebliche Änderungen und Aktualisierungen waren hingegen in den einzelnen Kapiteln selbst erforderlich; das gilt insbesondere für diejenigen Kapitel, die sich mit den Erkrankungen der einzelnen Organe und Organsysteme beschäftigen.

Die neuen Errungenschaften und neuen Erkenntnisse der medizinischen Forschung, die in dieser Neuauflage zu berücksichtigen waren, beziehen sich schwerpunktmäßig in erster Linie auf die erheblichen Fortschritte der apparativen Diagnostik, auf die vielfach verbesserten Möglichkeiten der Krankheitstherapie und auf die ursachenorientierte Einteilung der Krankheiten. Oberster Grundsatz bei der Aufnahme von Novitäten war für uns, wie auch früher schon, Neues jedoch nur dann zu berücksichtigen, wenn es bereits gesichertes Wissen darstellt, damit beim Leser als betroffenem Patienten keine verfrühten Hoffnungen geweckt werden. Darüber hinaus haben wir uns bemüht, auch in dieser Auflage der Tendenz des erfolgreichen Buches treu zu bleiben: der aufgeklärte Patient als Partner des Arztes.

Mannheim, im Juni 1984

Verlag und Herausgeber

PROSTATAERKRANKUNGEN

Unter *Prostatahypertrophie* versteht man eine gutartige Wucherung der Vorsteherdrüse, die durch Raumverdrängung zur Harnsperre führt. Die Bezeichnung Prostatahypertrophie ist insofern irreführend, als nicht einfach die gesamte Vorsteherdrüse sich vergrößert. Vielmehr wuchern bestimmte Drüsen im Bereich der hinteren Harnröhre, die sich dadurch auszeichnen, daß sie nicht nur vom männlichen, sondern auch vom weiblichen Geschlechtshormon des Mannes kontrolliert werden *(Prostataadenom)*. Im Alter führen wahrscheinlich Ungleichgewichte im Hormonhaushalt zur Wucherung dieses doppelt hormonabhängigen Vorsteherdrüsenanteils, doch ist die genaue Ursache der Prostatawucherung noch keineswegs bekannt.

Die gutartige Wucherung der Vorsteherdrüse tritt bei 80 % aller 60jährigen Männer auf und bleibt in der Hälfte der Fälle beschwerdefrei. Bei rund 40 % aller 60jährigen entstehen früher oder später und mehr oder weniger deutlich die Anzeichen einer Harnsperre. Dann ist die Drüsenwucherung so stark, daß die Blasenmuskulatur das Hindernis um die Harnröhre trotz Wandverdickung nicht mehr überwinden kann. Es entstehen Entleerungsstörungen der Harnblase, die unbehandelt schließlich zur Harnstauungsniere oder gar zum Tod durch Harnverhaltung führen können. In einem ersten oder Reizstadium besteht nur häufiger Harndrang; der Harnstrahl ist schwach und dünn; oft und auch nachts wird immer wieder eine kleine Harnmenge abgesetzt, doch ist die Blase zu diesem Zeitpunkt noch so gut wie vollständig entleerbar. Im zweiten Stadium erfolgt die Blasenentleerung nicht mehr vollständig, vielmehr bleibt jetzt jeweils eine Restharnmenge von mehr als 100 cm^3 zurück; dadurch erfährt der häufige, quälende Harndrang nun keine volle Erleichterung mehr, und der Rückstau führt häufig zur eitrigen Blasenentzündung. Im dritten Stadium wird die Harnröhre so weit eingeengt, daß die Blasenmuskulatur das Hindernis nicht mehr überwinden kann; daher läuft der Harn bei gesteigertem Blaseninnendruck ständig langsam und tropfenweise über; es kommt zur Harnstauungsniere und schließlich zum Nierenversagen (Abb. 1).

Die Prostatahypertrophie kann durch rektale Abtastung der Vorsteherdrüse recht frühzeitig erkannt werden, vor allem, wenn das Adenom sich unterhalb der Harnblase entwickelt hat (Abb. 2). Wächst die Wucherung in den Blasengrund ein, ist u. U. eine Harnblasenspiegelung erforderlich (Abb. 3). Wenn eine gutartige Wucherung der Vorsteherdrüse sicher diagnostiziert ist, besteht die Wahl zwischen einer Hormonbehandlung und der Entfernung der hypertrophierten Vorsteherdrüse. Die Hormonbehandlung geht von der Annahme gestörter Gleichgewichte aus und besteht in der Zufuhr abgewandelter männlicher, evtl. auch weiblicher Geschlechtshormone. Dauerheilung verspricht indessen nur die Operation; es kommt eine Ausschälung oder Elektroverschorfung der Vorsteherdrüse in Frage. Allzuspäte Operationen sind durch Blaseninfekte oder gar Rückstauerkrankungen der Niere belastet. Ein Operationsweg führt dicht über dem Schambein, ein anderer vom Damm her zur Vorsteherdrüse (Abb. 5). Inoperable Fälle können durch einen Dauerkatheter oder fortlaufende Selbstkatheterisierung behandelt werden.

Der *Vorsteherdrüsenkrebs (Prostatakarzinom)* ist bei Männern der häufigste Organkrebs. Er führt örtlich zu ganz ähnlichen Erscheinungen wie die Prostatahypertrophie, siedelt jedoch frühzeitig Tochtergeschwülste ins Knochensystem ab. Auch hier kann die Frühdiagnose durch rektale Austastung recht zuverlässig gestellt werden. Daher sollte jeder Mann über 45 Jahre einmal jährlich rektal untersucht werden. Der Prostatakrebs ist bei der Austastung als eine höckerige, harte Geschwulst zu fühlen. Zur Abgrenzung gegen die gutartige Prostatahypertrophie ist oft eine feingewebliche Untersuchung durch „Stanzbiopsie" nötig. Kennzeichnend sind ferner veränderte Blutserumphosphatasewerte. Zur Behandlung des Prostatakarzinoms stehen je nach Krankheitsstadium und Alter die Entfernung der Vorsteherdrüse, die Strahlenbehandlung, die Therapie mit gegengeschlechtlichen Hormonen und die Hodenexstirpation zur Verfügung („hormonelle Kastration").

Abb. 1 Harnstauungs
Prostatawuche

Abb. 4 Prostatakrebs m
Harnblase und

Abb. 5
Operative Entfernung
(Ausschälung)
der gewucherten Prostata

• *Reproductive Toxicology Review*

REPRODUCTIVE AND GENERAL METABOLIC EFFECTS OF PHYTOESTROGENS IN MAMMALS

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Key Words: Phytoestrogens, Mammalian reproduction, Reproductive hormones, Gonadal steroids, Luteinizing hormone, General Metabolism, Ovarian function, Reproductive neuroendocrinology.

INTRODUCTION

Historically, phytoestrogens were first investigated when it was noted that ewes that grazed Australian clover pastures for prolonged periods of time became sterile. It was found that the active agents in the clover that precipitated sterility were estrogenic (1). Later a similar phenomenon was observed to occur in the California quail during dry years, when phytoestrogen concentrations in available forage were increased (2).

Phytoestrogens are defined as plant substances that are structurally and functionally similar to the gonadal steroid 17β-estradiol (E_2) or that produce estrogenic effects (3). There are three main groups of nonsteroidal dietary estrogens. Phytoestrogens include the isoflavones (i.e., genistein, genistin, daidzein, biochanin A, formononetin, and prunetin) and the coumestans (i.e., coumestrol and 4'-o-methylcoumestrol). Mycoestrogens of the resorcylic acid lactone group (i.e., zearalenone and zearalenol) are also commonly found (4). The structural similarity between these substances, endogenous mammalian estrogens (E_2 and estrone), and potent synthetic estrogens (diethylstilbestrol) have been studied (Figure 1). Isoflavones, the monocarboxylic derivatives of the 15-C flavones, and coumestans contain central structures of 15 carbons. Both of these groups are derivatives of 3-phenylchroman (Figure 1) and thus may be considered a single family of compounds (5). The fungal resorcylic acid lactones and endogenous estrogens possess central structures of 17 carbons.

The similarity among these compounds has led investigators to study the possibility that phytoestrogens might act on physiological processes and behavioral patterns to alter reproductive performance (3). If reproductive effects occur, then these compounds might have a role in the evolutionary success of herbivores, perhaps making the difference between survival and extinction for some species. It is possible that phytoestrogens, through mimicry of endogenous animal estrogens, function as defensive substances by which plants diminish the fertility of herbivores which feed on the plants (6). In effect, the phytoestrogens may be seen as one of the many variables determining animal fitness for survival. This argument is supported by noting that animal species differ in their sensitivity to phytoestrogens (7). Some species are relatively resistant to the estrogenic effects of these compounds, while others may suffer sterility as a result of prolonged ingestion of phytoestrogens. We have hypothesized that phytoestrogen-induced physiologic and behavioral effects in mammals are significant factors in the reproductive and therefore evolutionary success of the consuming species. We have initiated our analysis of this broad hypothesis by reviewing the available data relevant to the reproductive and general metabolic effects of phytoestrogens in mammals.

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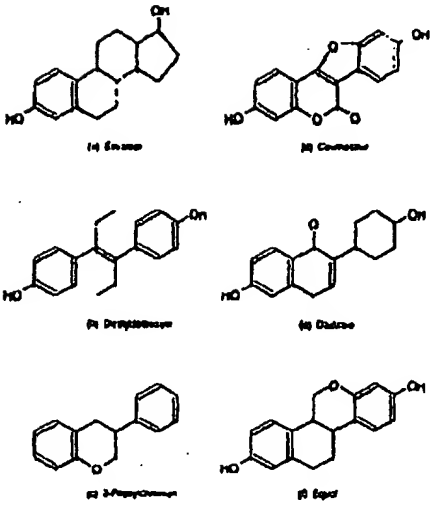

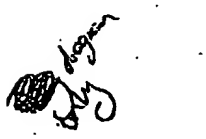

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Table 1. Some common plants that contain estrogenic substances

Alfalfa	Coffee	Oats	Rice
Anise	Date Palm	Orchard grass	Rye
Apple	Fennel	Palmetto grass	Sage
Barley	French Beans	Parsley	Sesame
Blue grass	Garlic	Peas	Soybean
Carrot	Green Beans	Pomegranate	Soya sprouts
Cherry	Hops	Potato	Wheat
Clovers	Liquorice	Rape	Yeast
	Marijuana	Red Beans	

PHYTOESTROGEN EXPOSURE

Sources of phytoestrogens

Phytoestrogens are produced by numerous Leguminosae and grasses, including many plants commonly consumed by man and livestock (Table 1). The estrogenic components are found in differing amounts in all parts of the plant, including the seeds, the flowers, the leaves, the roots, and the fruits. Concentrations in each tissue depend on plant type (4,8).

Of particular interest regarding possible human exposure is the presence of phytoestrogens in marijuana and coffee. It had long been suspected that the estrogenic effects of marijuana were due to Δ^9-tetrahydrocannabinol (THC), the major psychoactive compound. Smoking of marijuana significantly suppresses luteinizing hormone (LH) levels during the human menstrual cycle and shortens both the menstrual cycle and the luteal phase (9). Since these results agree with observations in ovariectomized rhesus monkeys injected intramuscularly (i.m.) with THC, it was assumed that the menstrual cycle effects of smoke inhalation would be exclusively due to the THC content of the smoke (10). However, crude marijuana extract and condensed marijuana smoke compete with estradiol for estrogen receptors in the uterus of rats, while in vitro studies detected no binding of cannabinoids to estrogen receptors (11). These findings show that marijuana contains estrogenic substances that may be affecting reproductive processes via cannabinoid-independent mechanisms. Furthermore, apigenin, a derivative of flavonoid phytoestrogens found in crude marijuana, is a moderately potent inhibitor of estradiol binding to uterine estrogen receptors (11). Differentiation between the suppressive effect of THC on LH and the estrogenic effects of marijuana *per se* remains unclear.

Another plant product which is commonly ingested for pleasure rather than nutrition is coffee. Like marijuana, coffee contains weakly estrogenic constituents, evidenced by the estrogenic effects of increased uterine-to-body weight ratio and total uterine protein content following administration of coffee extracts by gavage (12). Ultraviolet absorbance spectroscopy suggests that whatever this active compound may be, it does not belong to one of the three major classes of dietary estrogens (e.g., flavonoids, coumestans, or resorcylic acid lactones). Thus, coffee may contain an estrogen precursor that requires metabolic activation or a structurally unrelated estrogenic compound.

Metabolism, distribution, and clearance

The relative potency of a phytoestrogen depends upon the target tissue, functional state of the target tissue, the animal species involved, and the route and pattern of delivery. In addition, the fami-

ites of estrogenic compounds that occur in plants can be modified by metabolism within the herbivore or even by gut flora prior to uptake. Dietary isoflavone phytoestrogens undergo bacterial modification in the gastrointestinal tracts of animals to yield equol, a weak, nonsteroidal phytoestrogen (8,13,14). Following ingestion of estrogenic plants, a temporary 50- to 1000-fold increase in urinary equol takes place, while insignificant traces of the initially consumed phytoestrogens appear in the urine. Noteworthy is that the major urinary product following the consumption of genistein and biochanin A is p-ethyl phenol, and formononetin consumption yields both daidzein and equol as the major urinary products (4). Furthermore, gut microflora (14) convert daidzein to equol which in turn is absorbed and enters the enterohepatic circulation. Notably, it appears that not all people have the ability to convert other isoflavones to equol. This may be due to the absence of bacteria capable of the conversion of precursors to equol (as is the case in the sterile gut of newborns), the composition (subpopulations) of intestinal microflora present, the intestinal transit time, pH, or redox potential. These factors may be influenced by diet, host immunity, medication use, etc.

Receptor activity and interaction with endogenous estrogens

Phytoestrogens exhibit binding to endogenous estrogen receptors. Binding of phytoestrogens to estrogen receptors is supported by the finding that the larger the dose of phytoestrogen given an organism, the greater the displacement of bound tritiated (^{3}H) E_2 (15). It has also been reported that at very high dosages, all phytoestrogens exhibit more than 80% competitive binding to renal tumor cytosolic estrogen receptors (16). The structural requisites for estrogen receptor binding are met by phytoestrogens. For example, equol possesses a potency on the order of 10^{-3} the estrogenic activity of E_2 and contains a phenyl substituent also present in E_2 and in DES (Figure 1). The substituent considered to be a requirement for estrogenic activity is a hydroxyl group in the same position as the hydroxyl group in the benzene ring of E_2 (14). Another structural similarity which facilitates estrogen receptor binding activity of equol and other phytoestrogens is that the distance between C-3 and C-17 in E_2 is about equal to that between the two hydroxyls in equol.

Considering the large quantities of phytoestrogens ingested by many mammals including man, functionally significant estrogen receptor occupancy by phytoestrogens occurs. Since no phytoestrogen has receptor affinity equal to that of E_2 and the degree of DNA stimulation due to phytoestrogens appears to be substantially less than that evoked by E_2 (8), phytoestrogen actions could be either estrogenic or anti-estrogenic. In a relatively hypoestrogenic individual, receptor occupancy by weak (exogenous) estrogens would likely produce estrogenic effects, while in a normally estrogenized individual, large amounts of weak estrogens might diminish the effective estrogenic activity by competition with E_2.

REPRODUCTIVE EFFECTS IN MAMMALS

Phytoestrogens have been shown to influence virtually every aspect of the mammalian reproductive process via effects on the morphology and physiology of reproductive organs and alteration of sexual behavior. The changes may be reversible or irreversible, depending on the duration and dose of exposure to the phytoestrogens.

Cervix

A pubertal pattern of cell differentiation has been noted in ewes rendered sterile by chronic ingestion of phytoestrogens (17). Among these changes, the cervix assumes a uterine pattern. Folds present in the cervix fuse, resulting in loss of cervical crypts, and the cells of the lamina propria become like those of the uterine stroma. Furthermore, glands having histochemical reactions reminiscent of uterine glands become plentiful in the cervix. Such an increase in abnormal glands may be responsible for the different composition which the cervical mucus takes in sheep with "clover disease." At low phytoestrogen dosage, the cervical mucus has a lower viscosity, not due to a higher water content, but rather due to a decreased concentration of glycoprotein — the component of mucus that affords its consistency. The level of glycoprotein seems to respond to the duration of exposure to the phytoestrogen rather to the dosage of the agent. This change in the cervical mucus compounds the anatomical compromise of the cervix such that the cervical reservoir for sperm in the ewes is greatly reduced. Since sperm recovered from the cervices of clover-affected ewes exhibit decreased motility (17), it appears that the phytoestrogen effect makes the mucus relatively "hostile" in the classic sense of cervical factor infertility. Such spermatoxicity is not understood in general nor in this specific case.

At higher phytoestrogen dosage, both higher volume and water content of cervical mucus are

[redacted]

observed in ewes (17,18), thus indicating that both cervical glycoprotein production and water excretion in the mucus are affected.

The cervical effects of phytoestrogens likely depend upon estrogen receptor mediation. In ewes, phytoestrogen treatment increases the rate of protein and glycoprotein synthesis and the number of estrogen binding sites in the cervix, but binding affinity remains unchanged (19). This finding implies that exogenous estrogen not only occupies the available binding sites, but stimulates the local production of more sites. Such receptor "up-regulation" may make the tissue more sensitive to estrogen action, and, if estrogen exposure continues, the cervical alterations would become more exaggerated.

Uterus

Pronounced uterine effects of phytoestrogens are also observed. The most notable uterine change that occurs is a marked increase in its weight relative to body weight, which constitutes the classic bioassay for estrogen action. A dose-dependent uterine weight increase is precipitated by acute administration of an extract of the Indian herb *Achyranthes aspera* in rats and hamsters at contraceptive dosage (75 mg/kg) and with as little as 1/20 this dosage (20). Similar results have been observed in mice, rats, and hamsters with only 1/40 contraceptive dose of ferujol extract (21). Stob (4) suggests that this hypertrophy of the uterus is the result of "typical estrogenic mechanisms," implying estrogen-receptor mediation. However, a more complex response to daily s.c. injection of female lambs with the phytoestrogen β-sitosterol has been reported, in which uterine weight increases for the first two weeks of treatment but markedly decreases over the next six-week period (22). Plausible explanations for such biphasic results include receptor "down regulation" and induction of metabolic enzymes with enhanced clearance of β-sitosterol. Similar results were obtained using ovariectomized ewes as the model (23).

Another manifestation of the uterotropic effect of phytoestrogens is seen in ewes suffering from infertility due to prolonged exposure to these agents. A marked increase in activity of some uterine enzymes and uterine DNA, protein, and glycoprotein synthesis occurs in such sheep (19). This observation indicates that at least a portion of the uterine weight gain is true hypertrophy rather than simply edema. At the same time, lower levels of lipids within the uteri of sheep fed phytoestrogen suggest inhibition of synthesis or increased utilization of lipids within this organ (22). Thus phytoestrogens may be affecting different enzymes in different fashions, stimulating the activity of some while blocking the action of others. It is noteworthy that the uterine RNA-to-DNA ratio decrease that occurs following ovariectomy is smaller in clover-affected than in normal ewes. This response is accompanied by less regression of the uterus in clover affected ewes than in controls. These findings indicate that phytoestrogenic action may be mediated via differentiations similar to those induced by hormonal steroids during fetal development (24).

Gross structural lesions of the uterus may also result from phytoestrogen exposure and could account for some instances of permanent sterility. Lesser lesions entail the proliferation of cystic endometrium, myometrial fibrosis, and endometrial fibrosis (13). These lesions could certainly compromise normal implantation of the conceptus. The most severe structural failure, complete uterine prolapse, is known to occur in some species following ingestion of some dietary estrogens (mycoestrogens) and obviously disrupts the reproductive process.

It is not clear whether phytoestrogens play any role in pregnancy wastage, but some plant preparations have been used as abortifacients. *Achyranthes aspera*, a common Indian herb claimed to possess abortifacient activity, did induce abortion in mice and rabbits, but failed to show similar effects in rats (20). It is uncertain whether a phytoestrogen is the active agent of *Achyranthes* that brings about abortion, but support for that possibility derives from the finding that miroestrol, a phytoestrogen from a legume tree root, is used by Burmese and Thai women in plant extract form to induce abortion (25). The mechanism for such an abortifacient action of these compounds is unstudied and any effects of phytoestrogens on uterine contractility *per se* have not been determined in either the gravid or nongravid state.

Phytoestrogen effects on uterine function may relate to alterations in activity of several enzymes. Under normal circumstances, oxidative enzymes in the uterus show slight reactions in the endometrium and uterine glands, but after administration of β-sitosterol, these weak reactions are curtailed (22). Such an inhibition of oxidative enzymatic activity in the uterine endometrium and glands may reduce local energy production due to an inability to replenish NAD+ and NADP+. This circumstance would diminish the ability of the uterus to contract and might decrease secretory capabilities of the uterine glands.

Alkaline phosphatase in the uterine tissue of ewes also responds to β-sitosterol in a biphasic pat-

tem. Alkaline phosphatase activity increases over the first two weeks of daily β-sitosterol injections and decreases over the second two weeks of injections (22). This disturbance in alkaline phosphatase activity may alter cell permeability and transport of nutrients by uterine cells.

Acid phosphatase activity in the uterus decreases with increasing dose and time of daily β-sitosterol treatments over an eight-week span (22). Such an inhibition would decrease free phosphorous, and may relate to the more general observation of decreased plasma phosphorus levels in exposed animals.

Uterine cholinesterase activity also decreases following β-sitosterol treatment, as evidenced by its diminished activity towards acetylthiocholine (22). This inhibition of activity is accompanied by a downward shift in sodium ion transport and decreased sodium in the uterine luminal fluid. It is not clear whether effects on sodium transport and cholinesterase activity are coincidental or truly associated processes in this instance.

Ovaries

While many anatomical effects of phytoestrogens have been described, physiologic changes in the reproductive tract are more subtle, but perhaps more consequential. Ovarian cyclicity may be disrupted by phytoestrogen exposure in mammals and birds (2,14,25,26), but interruption of ovulation due to short-term phytoestrogen ingestion is reversible (26). It is plausible that human vegetarians may have ovulatory dysfunction but suffer no other obvious physiologic abnormalities due to their diets (14). Abnormalities of ovulation may be due to direct ovarian actions since administration of β-sitosterol to ewes inhibited follicular development and altered the size distribution of follicles (22). Follicles were observed to show degeneration with intrafollicular hemorrhage and the development of shrivelled oocytes with lipid inclusions. The suggestion of a direct ovarian action of phytoestrogens in perturbing follicular maturation may be supported to some extent by a study which showed that in rats intraperitoneal administration of an extract from a plant species known to contain high concentrations of phytoestrogens inhibited follicular maturation (26). Obviously, these studies cannot distinguish between direct ovarian and indirect effects on follicular growth.

More direct evidence that the follicle may be a site of phytoestrogen activity derives from in vitro cultures of bovine granulosa cells. In this system, lower dosages of genistein and biochanin A increased progesterone synthesis while higher dosages inhibited progesterone synthesis (27). Since progesterone is essential in the establishment and maintenance of pregnancy, such an inhibition of progesterone production would be a plausible explanation for both failure of conception and early pregnancy wastage.

The possibility that phytoestrogens might be toxic to oocytes or early embryos was suggested in a single study (7). Mice fed coumestrol and then mated produced degenerate embryos exhibiting unevenly distributed cytoplasm and lack of symmetry in size among blastomeres, suggesting alterations in cleavage rates. Extensive vacuolization found in the ova also suggests that failure of fertilization of these ova may account for part of the observed decrease in litter size in mice fed coumestrol.

The activities of two ovarian enzymes appear to be influenced by phytoestrogens. First, low doses of phytoestrogen inhibit 17,20-lyase in bovine granulosa cells (27). This effect could profoundly alter the pattern and capacity of the steroidogenic pathways within the follicle or corpus luteum. The precise mechanism by which this effect occurs is unproven. Second, alkaline phosphatase in the ovaries is affected by phytoestrogen exposure (22). While the overall alkaline phosphatase activity is about equal in the ovaries of β-sitosterol-treated and control ewes, the control ewes show an intense reaction in the zona pellucida with a weak reaction in the interstitial tissue. Treated ewes exhibit an opposite response. Thus, a reversal of activities is seen where phytoestrogen is acting both to stimulate and to inhibit the same enzyme in two different sites within the ovary. While a mechanism for this action is not known, such changes in the activities of ovarian enzymes might compromise ovulation and increase the incidence of follicular degeneration in animals treated with phytoestrogens.

CNS/pituitary

Some phytoestrogen effects on ovarian function appear to result from indirect action on the secretion of gonadotropic hormones (7). In this context, there are four possible mechanisms of phytoestrogen action: 1) they are E_2 agonists, 2) they are E_2 antagonists, 3) they act as both E_2 agonists and antagonists, and 4) they act in a nonestrogenic capacity. Available information best supports the third of these possibilities (mixed agonist–antagonist effects). The site of phytoestrogen action could be the CNS (especially hypothalamus), the pituitary, or the gonad (see previous section).

[redacted]

The effect of intraperitoneal injection of phytoestrogen-rich *Dieffenbachia amoena* extract in rats on LH, follicle-stimulating hormone (FSH), prolactin (PRL), progesterone, and E_2 have been studied (26). In treated rats, levels of LH, FSH, and progesterone increased for doses of 2.5, 5.0, and 10.0 mg/kg of extract, while the levels of PRL and E_2 decreased at the same dosages. Progesterone levels showed a biphasic response, increasing at low doses of the extract (26), but decreasing at higher doses (27). Since no obvious single mechanism would explain all of these pituitary and ovarian hormonal changes, the extract may contain more than one endocrinologically active substance, or more than one site or mechanism of action might be involved.

There are data to suggest that phytoestrogens act both at CNS and pituitary levels to alter gonadotropin secretion. In both ovariectomized ewes (23) and intact clover-affected ewes (17), the best explanation for the impairment of gonadotropin secretion was a hypothalamic/CNS action. In particular, in clover-affected ewes, an LH surge could not be elicited by exogenous E_2 administration (consistent with loss of positive feedback), but the LH secretory response to exogenous gonadotropin-releasing hormone was normal (17), suggesting no pituitary effect. Our own data (28) show that acute phytoestrogen administration can alter GnRH-induced LH secretion in ovariectomized rats and thus suggest that the pituitary may be a site of phytoestrogen action in other situations.

Interactions between reproductive effects of phytoestrogen exposure and photoperiod in seasonal breeders have been investigated. In normal intact ewes, the frequency of LH pulses and plasma LH concentration are higher during breeding season than during anestrus season. In clover-diseased ewes, the frequency of LH pulses and LH concentration during breeding season are nearly the same as in normal ewes. In contrast during anestrus season, these LH pulse parameters remain at the high level of breeding season in clover-affected ewes, rather than decreasing as in normal ewes (18). These results suggest that a dissociation of normal photoperiod controls from the LH pulse generator may result from prolonged phytoestrogen exposure.

In ovariectomized ewes given estradiol implants, LH pulse frequency and amplitude vary seasonally, rather like the pattern seen in intact ewes. This seasonal variation in LH pulse frequency in ovariectomized ewes could depend upon extraovarian steroids from the adrenal glands, other intrinsic photoperiod-dependent CNS functional changes, or dietary estrogens. Results from one study suggest that dietary coumestrol decreases the amplitude of LH pulses but fails to affect the frequency of LH pulses or FSH concentrations during the breeding season (23). During anestrus, coumestrol does not alter any of these variables. Thus, coumestrol could only be partially responsible for the seasonal decrease in LH pulse frequency in ewes.

Sexual behavior

Changes in sexual behavior due to phytoestrogen exposure parallel the known physiologic effects. Clover-diseased ewes are slower than normal ewes to exhibit estrus behavior in response to either a single or several daily doses of E_2 (17,29,30). Accompanying the delayed estrus is a retarding of the first mount of the ewes by the ram, although the number of days on which the ewes allowed the ram to mount them does not significantly differ from controls. A delay of estrus in mice fed coumestrol also occurs (7), implying an antiestrogenic effect.

Apparent defeminization of the sexual behavior response following consumption of phytoestrogens is displayed by clover-affected ewes. These ewes show aggressive behavior, such as challenging and head hunting of rams and other ewes, sooner than control ewes following administration of testosterone (17). At the same time the ewes are slower in showing female behavior, such as standing to be mounted by a ram. Furthermore, clover-affected ewes exhibited less soliciting behavior than normals. However, the number of ewes that stood to be mounted decreased equally over the five-week period during which daily testosterone injections were given (30). Relative to controls, clover-diseased ewes exhibit a significantly greater degree of courting behavior 28 but not 21 days following treatment with testosterone. Other courting behaviors that are less hormonally dependent, such as anal and genital sniffing by the ewes, are not altered (17,30). While mechanisms for these behavioral effects are not known, we do know that females and males have similar numbers of estrogen binding sites in the hypothalamus, but estrogen-receptor complexes appear to have shorter nuclear acceptor occupancy in males than in females (31). Behavioral changes in clover-affected ewes could result from a change as simple as a decrease in nuclear acceptor occupancy by estrogen-receptor complexes.

E_2 causes a dose-dependent increase in the incidence and duration of hormone-dependent behaviors in ewes (Table 2), whereas E_2 has no effect on hormone-independent behaviors (30). The E_2

Table 2. Estradiol-dependent and -independent behaviors in ewes

Hormone-dependent behaviors	Hormone-independent behaviors
Active soliciting	Squatting
Standing for mounting	Looking over shoulder
Allowing ram to mount	Tail fanning
	Kicking

induced behaviors occur less in phytoestrogen-affected ewes than in normals, while E_2 independent behaviors occur with equal frequency in control and clover-diseased ewes. Since general behavior appears normal but female sex-specific behavior is compromised in phytoestrogen-treated ewes, reproductive success could be compromised on a behavioral basis. The relationship of phytoestrogen-induced anatomic changes in the external genitalia and sexual behavior is not defined, but coital biomechanics could be altered as a result of such end-organ effects. While vulvar and vaginal hypertrophy has been noted in various animals, masculinization has been observed in ewes (17) with clitoromegaly and fusion of the ventral commissure. Upon removal from estrogenic pasture, these changes do not reverse and could, therefore, permanently alter sexual function.

Phytoestrogenic effects in males appear to be consistent with expectations for exogenous administration of bioactive estrogen. Coumestrol increases teat length in wethers (23) and stimulates mammary hypertrophy in intact males. Rams grazed on estrogenic clover have reduced sperm counts (14), but it is not clear whether fertility is affected.

GENERAL METABOLIC EFFECTS IN MAMMALS

Protein synthesis

Some data suggest that phytoestrogens affect levels of plasma proteins. The effects of β-sitosterol on plasma concentrations of albumin, alpha-globulin, beta-globulin, gamma-globulin, and fibrinogen have been studied (32). Normal functions of these proteins are indicated in Table 3 (33). Even though total plasma protein concentration in mice is unaffected by s.c. administration of β-sitosterol, daily 25 to 100 μg injections of the agent increase four of the plasma proteins, but significantly decrease the gamma-globulin complex. The mechanisms of action of phytoestrogens in this system

Table 3. Plasma protein fractions affected by β-sitosterol*

Protein	Function	Effect of β-Sitosterol
Serum albumin	Regulation of blood volume; transport of fatty acids	Increase
Alpha-globulins	Transport of lipids, thyroxine, adrenal cortical hormones, and copper	Increase
Beta-globulins	Transport of lipids, iron, and hemes	Increase
Gamma-globulins	Act as most of the circulating antibodies	Decrease
Fibrinogen	Precursor to fibrin of blood clots	Increase

*(See reference 32).

are not established. It is likely that the phytoestrogens stimulate hepatic protein synthesis but inhibit production of gamma-globulins by lymphoid tissues. It is possible that the increased alpha-globulin concentration is a compensatory occurrence to erythrocyte count reduction that occurs following administration of β-sitosterol, thereby maintaining normal blood viscosity in the absence of normal erythrocyte concentration. The increase in the beta-globulin–fibrinogen complex appears to be correlated with its affinity for binding phosphorus. This affinity increases in response to β-sitosterol (32).

Enzyme activity of the liver

Phytoestrogens influence enzymes in nonreproductive as well as reproductive tissues. A relation between diet and synthesis of three enzymes in the liver of cheetahs has been shown. The affected enzymes, alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyltransferase, decrease in amount when cheetahs are taken off a diet high in soya bean content (thus high in phytoestrogen content) and given a chicken diet (13).

Inorganic plasma constituents

Phytoestrogens induce mineral changes in the blood. Subcutaneous injections of 25, 50, 75, or 100 μg of β-sitosterol increase calcium levels in mice, while doses of 5 or 10 μg of the phytoestrogen have no effect on calcium levels (34). Since E_2 inhibits bone mobilization, β-sitosterol may act by causing a decrease in E_2 levels via inhibition of gonadotropin secretion from the pituitary. Decreased ovarian E_2

production might then result in increased bone mobilization and increased serum calcium. Surprisingly, blood plasma phosphorus levels decrease following administration of 5 to 75 μg doses of β-sitosterol in mice, but show little change in response to a 100 μg dose (34). Decreases in phosphorus could be due to an enhanced rate of storage in an extravascular compartment, increased utilization of phosphorus by tissues, or increased renal clearance.

While β-sitosterol doses of less than 5 μg fail to change plasma magnesium levels, higher doses decrease plasma magnesium and increase both hepatic and intramuscular magnesium (34). Since magnesium is a smooth muscle relaxant, changes in uterine or tubal smooth muscle motility could result indirectly from this phytoestrogen action.

PHYTOESTROGENS IN HUMAN DISEASE

Deleterious roles

Phytoestrogens have been suggested to play both deleterious and beneficial roles with regard to illness. In the diets of cheetahs, phytoestrogens cause vascular hepatic lesions, in which the centrilobular and sublobular hepatic veins are partially or totally occluded (13). The possibility of human hepatic dysfunction must therefore at least be considered.

Vascular disease may be correlated with the consumption of dietary phytoestrogens (35). Coronary heart disease has been suggested to be associated with phytoestrogens consumed indirectly through the milk of cows; that is, the lactating cow consumes the phytoestrogens while grazing and, in turn, phytoestrogens in cow's milk are consumed by humans. One basis for this proposal is that phytoestrogens have more structural similarity to DES, a potent synthetic estrogen found to have atherogenic properties, than to endogenous estrogens such as E_2. The higher rate of coronary heart disease in human males might be explicable in part if human females are found to be better able to metabolize and excrete phytoestrogens.

Dietary estrogens could be a factor in cancer initiation in hormone responsive tissues, but no such instances have been demonstrated. Certainly phytoestrogens bind to both rat and human mammary tumor tissue and show competitive binding for mammary tissue E_2 receptors (15) raising the possibility of stimulation of estrogen-dependent neoplasms.

Beneficial roles

Estrogens have two opposing effects on cancer, depending on dosage. Large doses inhibit breast cancer tumor development and suppress growth of tumors already present, but small doses seem to promote tumor development and stimulate growth (36). This duality extends to phytoestrogens. Phytoestrogens may stimulate or inhibit tumor growth (8,14). One mechanism by which phytoestrogens may manifest their antitumor effects is blockade of estrogen receptors and uncoupling of receptor-mediated response. Thus the ability of endogenous estrogens to support tumor growth would be reduced. Indirect demographic support for a phytoestrogen-mediated reduction in cancers of hormone-responsive tissues might derive from the observation that women in countries consuming vegetarian diets have a lower incidence of breast cancer than in societies where a meat and vegetable diet is consumed (37).

Phytoestrogens may have antiviral and fungicidal properties (37), but a mechanism is not known. Support for the notion that this group of compounds could have such properties may lie in noting that the antifungal drug, ketaconazole, is also a potent inhibitor of some steroidal enzymes.

Plant estrogens have been implicated in the reduction of serum cholesterol levels in humans and animals with hypercholesterolemia. Such action is likely related to the role estrogens play in the metabolism and interaction of lipoproteins with regulation of cholesterol (8).

A final beneficial phytoestrogenic effect is alleviation of vasomotor symptoms in menopausal women. Historically the Chinese have used herbal medicine to treat "hot flushes." These herbal medications work as well as Premarin (an equine conjugated estrogen) in the mitigation of these symptoms in women with natural menopause (38). Similarly, the mycoestrogen, zearalanol, has been reported to reduce the incidence of hot flushes in women with surgical menopause (4). These effects would be consistent with the expected estrogenic properties of these compounds.

CONCLUSION

Phytoestrogens influence mammalian reproductive processes and can thereby compromise the reproductive success of individual mammals and possibly function as a selective environmental factor for populations. While phytoestrogens have a few propitious effects, the majority of the effects are noxious. These compounds act through their similarity to endogenous estrogens and compete with the endogenous estrogens for binding sites.

Short-term effects of phytoestrogens seem to result from their mixed agonist–antagonist effects on estrogen-mediated processes in mammals. Since long-term exposures can produce persistent, even permanent anatomic, physiologic, or behavioral changes, phytoestrogens must affect the differentiation of some reproductive tissues and irreversibly alter the integration of mammalian reproductive processes in susceptable species.

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(19) JAPANESE PATENT OFFICE

PATENT ABSTRACTS OF JAPAN

(11) Publication number: **62106016 A**

(43) Date of publication of application: **16.05.87**

(51) Int. Cl **A61K 31/35**
// C07D311/34

(21) Application number: **60245508**

(22) Date of filing: **01.11.85**

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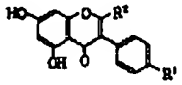
(54) **IMMUNO-SUPPRESSOR**

(57) Abstract:

PURPOSE: To provide an immuno-suppressor containing a specific isoflavone compound as an active component, having low toxicity and excellent immuno- suppressing activity and useful for the remedy and the prevention of relapse of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, etc.

CONSTITUTION: The isoflavone compound of formula (R^1 is OH or methoxy; R^2 is H, carboxyl or ethoxycarbonyl) is used as an immuno-suppressing agent. Concrete examples of the compound are 5,7,4'-trihydroxyisoflavone, 5,7-dihydroxy-4'-methoxyisoflavone-2-carboxylic acid, etc. The compound of formula has excellent immuno-suppressing activity and is useful for the remedy and prevention of relapse of human autoimmune diseases such as chronic active hepatitis, osteoporosis, etc. It is administered orally or parenterally at a dose of usually 200W1,000mg/day.



in newborn babies support an essential role for n-3 fatty acids in retinal development.[5]

The DHA content of erythrocytes is small compared with that of grey-matter, but the fatty acid composition of the erythrocyte membranes may indicate the fatty acid status of neural and perhaps other membranes. During the period of most rapid DHA accumulation in the developing rat, diet-induced changes in neural DHA are reflected in red blood cell DHA.[6]

Dietary n-3 fatty acids can also modify endogenous prostaglandin production and perhaps by this means influence uterine prostaglandins and gestation time. In the Faroe Islands, where birthweights are amongst the highest in the world with long gestation periods and rapid fetal growth, the intake of marine fat rich in n-3 fatty acids is high and erythrocyte DHA values in pregnant women were found to be almost twice those in normal individuals in other countries.[7]

The lower erythrocyte DHA found in patients on epoetin could be due to an increased requirement for this n-3 fatty acid as a result of increased red cell production, and this implies a deficiency of or a rate-limited production of DHA. However, plasma DHA values were not low, which raises the possibility of a defect of incorporation of this fatty acid into the membrane in patients on haemodialysis.

A low membrane DHA probably has little effect on red cell function and may be of minor importance in adults, although it is of interest that visual hallucinations have been described in patients on epoetin.[8] Unlike the adult, the fetus requires DHA in quantity for its developing nervous system, and haemodialysed patients do occasionally become pregnant. For reasons not fully understood, pregnancy in uraemia is associated with a high risk of premature labour and retarded fetal growth.[9] A lack of DHA would be detrimental to the fetus, and our results indicate that in a uraemic pregnant woman on haemodialysis, low quantities of membrane DHA could be one of the hazards to which the fetus is exposed. Because epoetin gives rise to even lower membrane DHA content, its use could increase the risk to the fetus: n-3 fatty acid dietary supplements are indicated.

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Dietary phyto-oestrogens and the menopause in Japan

SIR,—Lock, in an article on the menopause,[1] has discussed differences between Japanese women and women in western societies. Japanese women have a much lower frequency of hot flushes than women in Canada. Lock concluded that "cultural indifference to the hot flush in Japan" was unlikely to account fully for these findings.

Recently our Helsinki group studied, in collaboration with Japanese scientists, the diet and phyto-oestrogen excretion in Japanese women and men, and in a few children.[2] The women's mean age was 50·4 (SD 18·0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. We studied a group of three men, three women, and three children living in Kyoto and consuming the traditional diet, and in this group we measured the isoflavonoid genistein.[3] We found a very high excretion of phyto-oestrogens in urine. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15·5 μmol per 24 h in a man) and two other isoflavonoids, daidzein and equol (table). All these compounds bind to oestrogen receptors and have weak oestrogenic activity.[4] The excretion of the isoflavonoids in urine of the Japanese women was much higher than in American and Finnish women (table) (ref 4 and unpublished data) and as high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than those of endogenous oestrogens in normal omnivorous women consuming a western or oriental diet (table).

URINARY EXCRETION OF ISOFLAVONOID PHYTO-OESTROGENS AND ENDOGENOUS OESTROGENS IN JAPANESE OR ORIENTAL WOMEN, AND IN AMERICAN AND FINNISH OMNIVOROUS WOMEN

Urinary isoflavonoid or oestrogen	Japanese/ Oriental	American	Finnish
Genistein	3440 (n = 3)*	..	32·1 (n = 12)
Daidzein	2600 (n = 10)*	216 (n = 21)	40·5 (n = 12)
Equol	2600 (n = 10)*	62·8 (n = 21)	11·2 (n = 12)
Oestrone (postmenopausal)	4·48 (n = 9)†	..	4·48 (n = 10)
Oestradiol (postmenopausal)	0·76 (n = 9)†	..	0·94 (n = 10)
Oestriol (postmenopausal)	4·48 (n = 9)†	..	1·44 (n = 10)

All assays by gas chromatography/mass spectrometry in selected ion-monitoring mode with deuterated internal standards.[6,7] Women collected two to four 72 h urine samples 3–6 months apart and values are thus means of urinary excretion in individual subjects over 6–12 days. Results as geometric means in nmol/24 h
*Values from ref 2.
†Oriental postmenopausal women (recent immigrants to Hawaii) Same women as in ref 7, but oestrogens measured by new technique.[6]

The excretion of the isoflavonoids in urine was associated with intake of soy products such as *tofu, miso, aburage, atuage, koridofu*, soybeans, and boiled beans. All isoflavonoids are weak oestrogens and such high amounts could have biological effects, especially in postmenopausal women with low oestrogen levels. High levels of isoflavonoid phyto-oestrogens may partly explain why hot flushes and other menopausal symptoms are so infrequent in Japanese women.

Supported by grants from the Medical Research Council, Academy of Finland, and S. Juselius Foundation, Helsinki.

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J. Steroid Biochem. Molec. Biol. Vol. 41, No. 3-8, pp. 331-337, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

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DIETARY PHYTOESTROGENS AND CANCER: *IN VITRO* AND *IN VIVO* STUDIES

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Summary—Thirty postmenopausal women (11 omnivores, 10 vegetarians and 9 apparently healthy women with surgically removed breast cancer) were investigated with regard to the association of their urinary excretion of estrogens, lignans and isoflavonoids (all diphenols) with plasma sex hormone binding globulin (SHBG). A statistically significant positive correlation between urinary total diphenol excretion and plasma SHBG was found which remained statistically significant after elimination of the confounding effect of body mass determined by body mass index (BMI). Furthermore we found a statistically significant negative correlation between plasma SHBG and urinary excretion of 16α-hydroxyestrone and estriol which also remained significant after eliminating the effect of BMI. Furthermore we observed that enterolactone (Enl) stimulates the synthesis of SHBG by HepG2 liver cancer cells in culture acting synergistically with estradiol and at physiological concentrations. Enl was rapidly conjugated by the liver cells, mainly to its monosulfate. Several lignans and the isoflavonoids daidzein and equol were found to compete with estradiol for binding to the rat uterine type II estrogen binding site (the s.c. bioflavonoid receptor). It is suggested that lignans and isoflavonoids may affect uptake and metabolism of sex hormones by participating in the regulation of plasma SHBG levels and in this way influence their biological activity and that they may inhibit cancer cell growth like some flavonoids by competing with estradiol for the type II estrogen binding sites.

INTRODUCTION

Weakly estrogenic diphenolic compounds, belonging to the classes of lignans (Ligs) and isoflavonoids (Ifls), are excreted in large amounts in human (and animal) urine. Subjects consuming whole-grain products, seeds, fruits and berries (contains mammalian lignan precursors) and soy products (contains isoflavonoids, and lignan precursors) [1–6] have high excretion of these compounds. Up to now about 15 structurally different compounds were isolated and identified by combined gas chromatography–mass spectrometry (GC/MS) [structures and literature in 4, 6, 7]. Intestinal bacteria play an important role in the transformation of the plant precursors [2, 7, 8].

Lignan excretion in women is usually high in areas with low risk for breast cancer (BC) like North Karelia in Finland [4], and in vegetarians [4, 5, 9, 10] and low in women living in high-risk areas like Boston, U.S.A. [4, 5, 10]. In old women with BC in Boston the excretion was very low [10] and it was also relatively low in Finnish young women with BC [9]. On the other hand we also found low excretion of Ligs in Japanese women consuming traditional Japanese diet and having low BC risk. However these subjects excreted very high amounts of Ifls, particularly genistein (Gen) and daidzein (Daid) [11]. There is already evidence suggesting that both Ligs and Ifls are protective with regard to BC [12–17] and that Ifls may be protective with regard to prostate cancer (PC) [16, 18].

In the present study we continue to explore the link between the Ligs and Ifls, and hormone-dependent cancer and the possible mechanisms by which the cancer-protective effect of these compounds is exerted. The results obtained strongly suggest that these compounds have cancer-protective properties.

Proceedings of the 10th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, Recent Advances in Steroid Biochemistry and Molecular Biology, Paris, France, 26–29 May 1991.
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MATERIALS AND METHODS

Subjects and their diet

In this connection only some preliminary *in vivo* results with regard to plasma sex hormone binding globulin (SHBG), urinary estrogens, Ligs and Ifls for the last part of the "Finlandia" study involving postmenopausal women will be described. The groups studied were 11 omnivorous and 10 vegetarian women and 9 apparently healthy women with breast cancer (BC) treated with surgical removal of the breast (Stage I and II). Simultaneously with the collection of urine and blood samples, very careful dietary records during 5 days were obtained, once in winter and once in the summer time. The dietary differences were surprisingly small. Preliminary calculations showed that the vegetarians had higher intake of total fiber (22.7 g/day, geometric means) than the omnivores (16.6 g/day) and the BC patients (16.0 g/day) but this was statistically significant only when compared with the BC group ($P < 0.04$). No significant differences in dietary intake between the omnivorous and BC groups could be observed. Cholesterol intake was significantly lower in the vegetarians (omnivores vs vegetarians $P < 0.02$; BC vs vegetarians $P < 0.002$). Furthermore we found a statistically significantly higher intake of vegetable fiber in the vegetarians compared to the BC group (vegetarians 4.5 g/day and BC 2.3 g/day, $P < 0.03$). Complete dietary data will be published elsewhere.

Collection of blood and urine samples

The women collected 72-h urine samples and three different blood samples were drawn between 8 and 9 a.m. into heparinized tubes on the same consecutive days. The plasma was pooled and the samples were stored with 0.1% ascorbic acid and 0.1% sodium azide at −20°C until analyzed. In the present study the mean values for one winter and one summer collection period were used (6 plasma and 2 × 72-h urine samples for each subject).

Reference standards and deuterium-labelled compounds

The Ligs Enl, End, matairesinol (Mat), and the Ifls, Daid, Equol and *O*-desmethyl-angolensin (*O*-Dma) were synthesized and the preparation of the deuterium-labelled standards was carried out as described previously [lit. in 19]. The isoflavonoid Gen was a generous gift from Professor K. Kallela.

Cell cultures

Prior to the growth experiments the cells (HepG2 liver cancer and MCF-7 breast cancer cells, American Type Culture Collection, Rockville, Md, U.S.A.) were maintained in Dulbecco's modified Eagle's medium (DMEM) without phenol red supplemented with 10 mM of L-glutamine, 100 IU/ml of penicillin, 100 IU/ml of streptomycin, 1% (v/v) NEAA, and 15 mM Hepes (Boehringer Mannheim, Fed. Rep. Germany) with 10% fetal calf serum (FCS). Before the experiment the cells were detached by removing the medium and washing with ice-cold Ca- and Mg-free phosphate buffered saline (PBS) (Orion Diagnostics, Espoo, Finland) and trypsinization (trypsin 0.05%, EDTA 0.02%). 100,000 to 400,000 cells, depending on the size of the plastic petri dishes, were plated in the same medium but now with 5% FCS for two days. The medium was removed, cells washed with PBS, and fresh medium with 5% twice DCC-treated FCS added and incubated for a further 3 days. The preparation of the DCC-treated FCS was carried out as described [15].

After the final washing of the cells twice with ice-cold PBS and added fresh medium with 5% DCC-treated FCS, the cells received effectors in ethanol solution to a final concentration of not more than 0.1% ethanol. The cells were maintained at 37°C in a 100% humid atmosphere of 92% air and 8% carbon dioxide as a monolayer culture in Falcon's plastic petri dishes (9 cm dia.) or in dishes with six 2.5 cm wells. The effectors were added once per day and the medium changed every fifth day. Duration of experiments was 8–10 days. Cells were counted both manually in a Bürker chamber and using the Coulter counter industrial cell counter (Coulter Electronics Ltd, Luton, Beds., England). DNA was measured by fluorometry with a slight modification of the original procedure [20] using the Transcon 102 FN fluoronephelometer (Elomit Oy, Helsinki, Finland) and the results were expressed in pmol/mg DNA.

Cell cultures in metabolite studies and determination of enterolactone conjugates

In the metabolite studies with HepG2 cells, the cells, after the initial treatment described above, were first grown for four days as described and every morning Enl was added to a final concentration of 1 μM. After four days the medium was removed and the cells washed with

ice-cold PBS and fresh medium added. Thereafter the procedure was continued for another 4 days and 24 h after the last addition of Enl extraction was carried out as described [15]. The fractionation of Enl conjugates and their determination was carried out as previously described [21] with slight modifications [15]. To the final fractions 211.2 ng $^2H_6$-labelled Enl was added in 50 μl of ethanol, the solvent evaporated to dryness and the samples silylated. After trimethylsilyl ether derivative formation the solvent was evaporated to dryness, the residue was dissolved in a suitable amount of n-hexane and the quantitation carried out by GC/MS in the selected ion monitoring (SIM) mode as described [22].

In studies on the time course of Enl conjugation, 1 million HepG2 cells were plated and Enl added to a final concentration of 2 μM. Samples were taken at various time intervals up to 74 h. Medium was extracted with ethyl ether and the conjugates hydrolyzed with *Helix pomatia* extract as described [22] and the liberated aglycone extracted with ether and assayed by GC/MS.

Determination of SHBG in the medium

SHBG assays in the medium were carried out with a highly sensitive time-resolved fluoroimmunoassay (TR-FIA) using reagents provided by Farmos Ltd (Turku, Finland).

Studies of the binding of diphenols to the nuclear type II estrogen binding site

Adult ovariectomized rats were implanted with 20 μg of estradiol (E2) and 96 h after treatment uterine nuclear fractions were prepared. The various lignans and isoflavonoids were dissolved in Tris–EDTA buffer containing 20% ethanol and their ability to inhibit the binding of [^{3}H]estradiol (40 nM) to nuclear type II sites were assessed [23, 24].

Assays of estrogens, lignans and isoflavonoids in urine and SHBG in plasma

Ligs and Ifls were determined in urine by an isotope dilution gas chromatographic–mass spectrometric method recently described [19] combining the method with the estrogen profile method also described previously [22]. This allows simultaneous assay of 20 compounds. In the present study Mat and Gen were not assayed because the method did not include these two compounds at the time of analysis. Thus we determined 13 estrogens and the Ligs Enl and End, and the Ifls Daid, Equol, and *O*-Dma. SHBG in plasma was determined by the RIA kit provided by Farmos Ltd.

Statistical methods

The mean values presented are geometric means. In the statistical analyses the mean values for the winter and summer collection periods were used and when necessary logarithmic transformation was made because of skewness of the distribution of the results. The degree of univariate associations between two variables was estimated as Pearsons correlation coefficients (r). Partial correlations were calculated to eliminate the effect of body mass index (BMI) on the results. Correlation coefficients and partial correlation coefficients were calculated using the StatView II programme for Macintosh II (Abacus Concepts, Inc. Berkely, CA, U.S.A.).

RESULTS

SHBG, diphenols and estrogen 16α-hydroxylation

In the three groups of postmenopausal women the plasma SHBG values were statistically significantly highest in the vegetarians (70.3 nmol/l) ($P < 0.0002$) compared to the omnivores (31.1 nmol/l) and BC patients (34.8 nmol/l). The vegetarians had significantly higher urinary excretion of Enl, total Ligs, total Ifls, and total diphenols ($P < 0.05$–$P < 0.007$) compared to the two other groups (details to be published elsewhere).

We found a statistically significant positive correlation between urinary excretion of *O*-Dma, Enl, total lignans and total diphenols and plasma SHBG. However, this correlation was partially dependent on the fact that the vegetarians had significantly lower body mass (BMI = omnivores 26.1, vegetarians 22.4, BC 26.2). BMI showed a negative correlation with SHBG ($r = -0.580$; $P < 0.001$) in these subjects. After elimination of the confounding effect of BMI we still found a statistically significant positive association between the urinary excretion of *O*-Dma ($r = 0.421$), Enl ($r = 0.391$), total Ligs ($r = 0.382$) and total diphenols ($r = 0.400$) and plasma SHBG ($P < 0.05$ for all).

When studying the association between plasma SHBG and the excretion of individual urinary estrogens we found that there was no association between SHBG and urinary catecholestrogens. However, we found statistically

significant negative correlations between SHBG and urinary 16α-hydroxyestrone ($r = -0.448$; $P < 0.05$) and estriol ($r = -0.572$; $P < 0.001$). Partial correlation coefficients eliminating the linear effect of BMI on the results showed that these significant associations remained but were weaker ($r = -0.390$ and $r = -0.409$ for 16α-hydroxyestrone and estriol, respectively, both $P < 0.05$).

Stimulation of SHBG synthesis by enterolactone in HepG2 liver cell cultures

Concentrations of Enl between 0.5 and 10 μM stimulated SHBG synthesis by HepG2 human liver cancer cells in culture (Fig. 1). The maximal effect was found with 5 μM concentration and a toxic effect could be observed with concentrations above 10 μM (Fig. 1). 200 nM concentration of estradiol (E2) was needed to obtain a similar stimulation of SHBG synthesis as 2 μM of Enl. When E2 (200 nM) and Enl (5 or 10 μM) were combined they had additive effects on the synthesis (Fig. 1).

Metabolism of enterolactone by HepG2 liver cells

The conjugation of Enl by HepG2 cells was very rapid and within 10 h more than 95% was conjugated (Fig. 2). The relative concentrations of the different conjugates of Enl identified in the medium are shown in Table 1. The main conjugate was the monosulfate (EnlS) amounting to about 78% of the total.

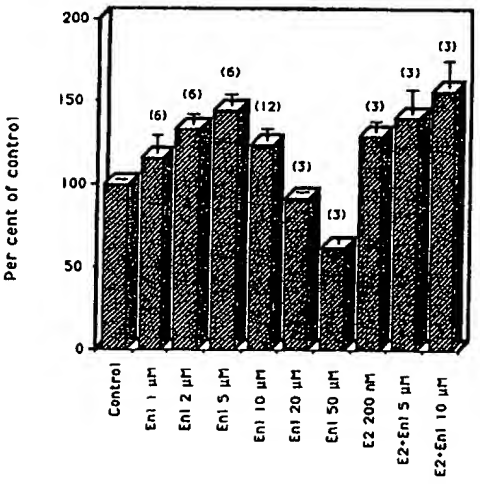

Fig. 1. Enterolactone stimulation of sex hormone binding globulin (SHBG) synthesis by HepG2 cells in culture in the absence and presence of estradiol. Number of experiments indicated on the top of the bars.

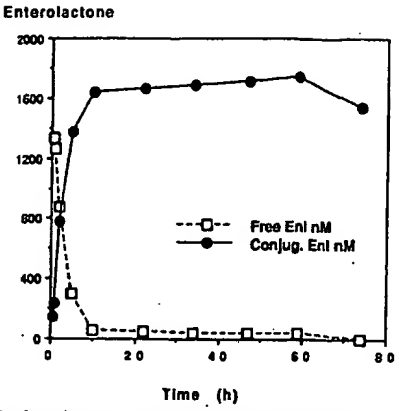

Fig. 2. Conjugation of enterolactone (Enl) (2 μM) added to cultures of HepG2 cells.

Binding of diphenols ot the nuclear type II binding site (bioflavonoid receptor)

Figure 3 (top) shows the binding of the two main mammalian lignans Enl and End to the nuclear estrogen type II binding site. In addition the binding of two plant lignans, matairesinol, which is the precursor of Enl [2, 7] and of isolariciresinol is shown. In the lower part of the figure it can be seen that daidzein and equol show significant binding but their precursor formononetin does not bind to the bioflavonoid receptor.

DISCUSSION

It has been proposed that a low rate of 2-hydroxylation and high rate of 16α-hydroxylation leads to a greater risk for BC and endometrial cancer. BC patients, women with genetic predisposition for BC and mouse strains with high incidence of BC have been shown to have high 16α-hydroxylation of estrogens [25–27]. Furthermore a parallel increase in *ras* proto-oncogene expression and of estradiol-16α-hydroxylation in human mammary terminal duct-lobular units by a carcinogen has been found [28].

Table 1. Distribution of conjugated metabolites of enterolactone in the culture medium 24 h after the last addition of enterolactone (1 μM) to the medium of HepG2 liver cancer cells in culture*

Fraction	%	Fraction	%
Unconjugated	0.12	Monosulfates	77.6
Monoglucuronides	6.29	Disulfates	5.33
Diglucuronides	6.82	Sulfoglucuronides	1.80
In other fractions	2.04		
Total	100.0		

*Means of two experiments.

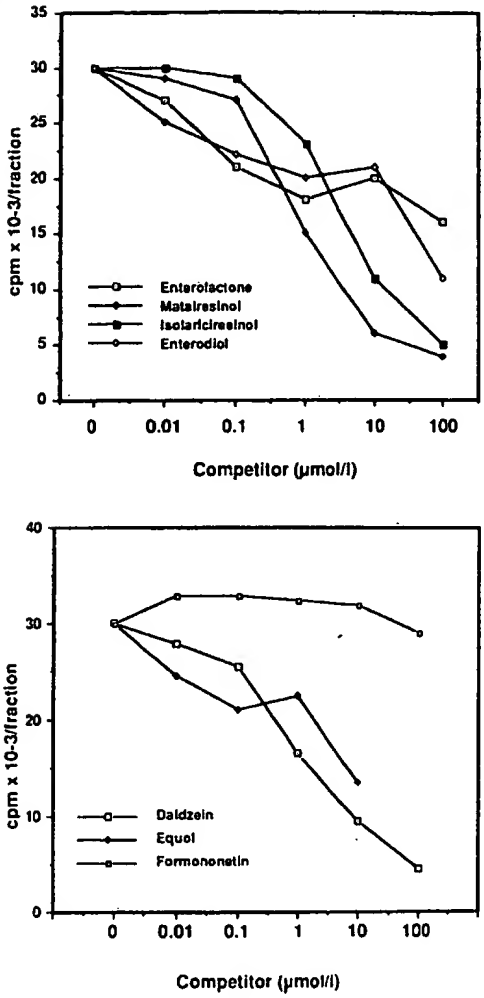

Fig. 3. Competition with [^{3}H]estradiol of lignans (top) and isoflavonoids with regard to rat uterine estrogen nuclear type II binding sites (bioflavonoid receptor).

Several earlier studies by others as well as our own seem to speak against the hypothesis that increased estrogen 16α-hydroxylation is a risk factor for BC, because all low-risk groups, compared to high-risk groups, have relatively more urinary 16α-hydroxylated estrogens, particularly if also the fecal estrogens are included. These observations have recently been discussed [16]. In addition we could previously not observe any increase in 16α-hydroxylated estrogen metabolites in urine of Finnish premenopausal women with BC compared both to omnivorous and vegetarian controls [29].

However, in the present study the old Finnish women showed statistically significant negative correlation between plasma SHBG and urinary 16α-hydroxyestrone and estriol in the whole material ($n = 30$) with the highest values of 16α-hydroxylated estrogens and lowest SHBG values in the women with BC and the omnivorous women. In the same material there was a significant positive correlation between urinary total diphenol excretion and plasma SHBG. All these associations remained statistically different despite elimination of the confounding effect of BMI on the results, although the associations were weaker. It has been shown in many studies that a low SHBG level means a higher metabolic clearance rate and uptake of sex hormones in many tissues including the liver, the principal site of estrogen 16α-hydroxylation. Postmenopausal women with BC have frequently central obesity and low SHBG levels [30–32] and we therefore suggest that some of the *in vivo* results obtained in BC patients showing increased estrogen 16α-hydroxylation may have been at least partly due to a low SHBG in the studied subjects. To our knowledge SHBG was never measured in these studies. In fact it is possible that increased cellular membrane permeability for nonpolar estrogens caused by different mechanisms may also in other tissues lead to increased 16α-hydroxylation.

Estradiol has been found to stimulate the *in vitro* synthesis of SHBG by HepG2 cells in culture, but the concentrations needed for significant increase in production are much higher (0.5–5 μM) than those occurring physiologically [33]. Our experiments show that only 10 times more Enl is needed to show the same stimulation of SHBG formation as that observed for E2. By relating SHBG synthesis to cell number and DNA it could be observed that this was not due to increased cell proliferation but to a true increase in synthesis. This was also confirmed by measuring intracellular SHBG after sonication of the cells.

The question arises whether the concentrations of Enl in the organism, particularly in the portal vein blood, are sufficiently high to have a stimulatory effect on SHBG synthesis. It is well known that estrogens administered orally, compared to parenteral administration, are much more effective in stimulating SHBG synthesis [34]. Enl enters the liver via the portal vein probably in much higher concentrations than those occurring in peripheral plasma. We know very little about the levels of Enl in plasma. Total Enl (free + conjugated) values in 4 women ranged between 0.7 and 5.3 nM [35].

Our own unpublished preliminary observations suggest that the concentrations are much higher in plasma of Finnish women and in vegetarians. We observed total Enl values between 15 and 70 nM and between 20 and over 1000 nM in omnivores and vegetarians, respectively. About 5–30% of the total occurs in the form of unconjugated Enl or in the sulfate form. As found for estrone sulfate, we believe that the sulfates of the lignans can be hydrolyzed at the cell membranes and have biological activity because of the abundance of intracellular sulfatases in the organism. Thus it is very likely that Enl in the free + sulfate form occurs in concentrations at least 10 times higher than those of unconjugated + sulfate-conjugated E2, particularly in the portal vein blood. This makes it very likely that these compounds may be involved in regulation of SHBG levels in plasma in agreement with the positive correlations observed in this and previous studies [5, 9] between excretion of lignans and isoflavonoids in urine and plasma SHBG.

Compared with MCF-7 BC cells [15], the HepG2 cells conjugate Enl as rapidly (Fig. 1 and Table 1), but less monosulfates and higher amounts of glucuronides and disulfates are formed. The monosulfates represented 78% of the total compared to 91% for the MCF-7 cells. Thus our preliminary results in plasma showing considerable amounts of sulfate-conjugated Enl in circulation are in good agreement with the *in vitro* metabolic results obtained with HepG2 cells.

Our results show (Fig. 3) that diphenolic lignans and isoflavonoids compete with E2 for the rat uterine nuclear estrogen type II binding site. These sites seem to constitute a component of the genome which regulates estrogen-stimulated uterine growth [23, 24]. Originally it was observed that some flavonoids like luteolin, quercetin and pelargonin inhibit E2 binding to this receptor and in this way uterine cell growth. They also inhibited growth of MCF-7 cells in culture, and *in vivo* E2 stimulation of immature rat uterus [36]. The structures of these flavonoids are very similar to those of the isoflavonoids. Luteolin, quercetin and pelargonin have to our knowledge not been identified in the human organism. However, Daid, Eq, Enl and End were all found in plasma, saliva and urine of human subjects and Enl, End and Eq in prostatic fluid [37, and unpublished, see above]. Now also Gen, Mat and *O*-Dma have been detected in plasma in our laboratory.

It was suggested that the isoflavonoids and flavonoids may all act synergistically inhibiting cell growth in malignant cells via the type II binding site [16] also called the bioflavonoid receptor [36, 38] or by inhibiting specifically the tyrosine protein kinase [16] the enzyme mediating the activity of many growth factors in the cell.

It is concluded that lignans and isoflavonoids may influence sex hormone metabolism and cancer by influencing plasma SHBG levels resulting in lower uptake and less biological activity of these steroids and by inhibiting growth and proliferation [13–15, 18] of hormone-dependent cancer cells.

Acknowledgements—This work was supported by grants from the Sigrid Jusélius Foundation, the Medical Research Council of the Academy of Finland, and The Finnish Cancer Foundations, Helsinki. The skilful technical assistance of Ms Sirkka Adlercreutz, Ms Anja Koskela, and Ms Inga Wiik is gratefully acknowledged.

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D10

PHYTOESTROGEN

DETECTION AND ESTIMATION OF OESTROGENIC CONSTITUENTS IN RED CLOVER

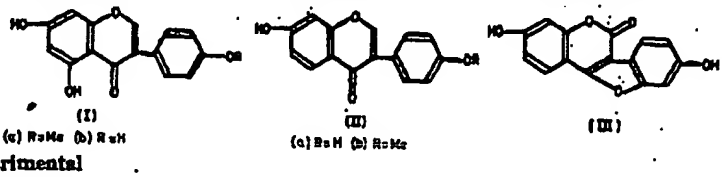
By E. WONG

A two-dimensional paper chromatographic method has been used for the separation and detection of all the known oestrogenic isoflavones and coumestrol in red clover. The presence of other isoflavone components in red clover is suggested. The concentrations of the individual oestrogenic constituents were determined by spectrophotometric measurement after separation by paper chromatography. Biochanin A has been found to be the predominant oestrogen in red clover, while formononetin (the other major isoflavone) is inactive.

Introduction

The weakly oestrogenic isoflavones biochanin A[1] (Ia), genistein[2] (Ib) and daidzein[3] (IIa) have been found in red clover (*Trifolium pratense*) as well as in subterranean clover (*Trifolium subterraneum*). Another isoflavone, formononetin (IIb), is also known to be present in red clover.[4] Coumestrol (III), the oestrogenic coumarin derivative first isolated from ladino clover (*Trifolium repens*), has also been reported to be present in red clover and other forages.[5] The oestrogenic activity of these compounds has been discussed in a recent review.[6]

Munford & Flux[7] have recently shown that the oestrogenic potency of New Zealand red clover is considerable. By the mouse uterine weight assay they estimated an equivalent activity of about 7 μg. of diethylstilboestrol per 100 g. of dry clover. In view of the relatively high oestrogenic activity and the known presence of several oestrogens in red clover, it became of interest to determine the relative contribution of these constituents to the total biological activity of the plant material. This paper describes a two-dimensional paper chromatographic method for the separation and estimation of the oestrogens in fresh red clover extracts.



Experimental

Plant material

Red clover plants (New Zealand late-flowering strain of the Montgomery variety) were sampled on two occasions during the growing season. Samples were taken in early Spring (October 1960) (Sample I) and in late Summer (March 1961) (Sample II). Freshly harvested herbage was separated into leaves and stem and petioles as soon as possible after cutting and immediately soaked in 95% ethanol.

Extraction of plant materials

A 50-g. sample was macerated in 250 ml. of 95% ethanol in a Waring Blendor. The resulting mixture was refluxed for 15 min., filtered, and the residue re-extracted with 250 ml. of boiling ethanol for 5 min. The combined filtrate was concentrated under reduced pressure and brought to 200 ml. of 70% aqueous ethanol by adding water. The aqueous alcoholic suspension was then extracted four times (total 250 ml.) with light petroleum (boiling range 60–65°). The petroleum extract was discarded and the aqueous alcoholic phase concentrated under reduced pressure. The residue was suspended in 100 ml. of water and extracted with four 100-ml. portions of ether. The combined ether extracts were evaporated to dryness to yield the ether-soluble fraction. The following amounts of ether-soluble materials were obtained from the various fractions: Sample I: leaves 220 mg., stems and petioles 105 mg.; Sample II: leaves 296 mg., stems and petioles 66 mg.

Paper chromatography

The ether-soluble fraction was taken up in ethanol usually at a concentration of 10 mg./ml.

Aliquots of 200 μl. were used for two-dimensional descending chromatography on Whatman No. 3MM paper previously washed with aqueous acetic acid (50%) followed by ethanol. The washings were carried out by downward irrigation of solvents for a total period of 2–3 days as for chromatography. The single phase system benzene–acetic acid–water (125 : 72 : 3)[8] was used for the first direction. This system has the advantage of moving contaminating green pigment and lipid material near the solvent front and gives a very good distribution of the polyphenols.

Aqueous ammonia (2N) was found to be the most suitable solvent for the second direction. Although substances such as flavonols are unstable in this alkaline solvent system, the oestrogens under consideration and most of the other components are not affected (but with biochanin A at high concentrations a slight visible brown 'beard' is usually observed just in front of the spot probably due to partial degradation).

The individual spots on the chromatogram were located by ultra-violet light (Hanovia 16, 365 mμ) and with spray reagents, diazotised sulphanilic acid[9] being the most suitable. The isoflavones were identified by comparison with synthetic materials.[10] Coumestrol was identified by comparison with an authentic sample. These constituents were found to be distinctly separated from one another and from other phenolic spots on the two-dimensional chromatogram. With the present solvent system, a distortion of the solvent front in the second direction was invariably observed. The appearance of the isoflavones and some other constituents on a typical chromatogram is shown in Fig. 1.

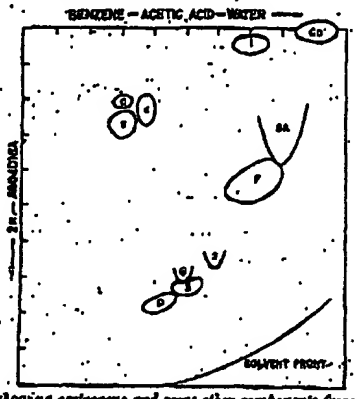


FIG. 1.—*Paper chromatogram showing oestrogens and some other components from red clover (not all spots shown)*

BA = biochanin A
F = formononetin
G = genistein
1, 2, 3 possibly isoflavones
D = daidzein
C = coumestrol
Cr = chlorophyll
4, 5 unknowns

Spectrophotometric measurements

The isoflavone spots on the chromatogram after location with ultra-violet light were eluted with ethanol for about 24 h. with occasional shaking. The identities of the isoflavones were confirmed by measurements of their ultra-violet spectra in ethanol, ethanolic NaOH (~0·01N)

Table I

Ultra-violet absorption data for isoflavones

	$\lambda_{max.}$ (mμ)			$E^{1\%}_{1cm.} \times 10^{-2}$ (max. in ethanol)
	Ethanol	0·01N-Ethanolic sodium hydroxide	0·1% Aluminium chloride in ethanol	
Biochanin A	263	276	275	1·23
Genistein	263	277	275	1·32
Formononetin	250	260	250	1·01
Daidzein	250	260	250	1·00

and ethanolic $AlCl_3$[12] (0·1%) with a Beckman D.U. spectrophotometer. The concentrations of the individual isoflavones were obtained from the optical densities at the absorption maxima. Standard solutions of the pure isoflavones were chromatographed as described for the extracts and eluted and measured in a similar manner to determine the recoveries obtained. Absorption maxima and $E^{1\%}_{1\,cm}$ determined are given in Table I.

Results and discussion

Detection of oestrogens

The two solvent systems used were found very satisfactory for the separation of the oestrogenic constituents of red clover by paper chromatography. Many other solvent systems commonly used for polyphenolic compounds[11, 12] were tried but were unsuitable. The isoflavones streak badly in aqueous acetic acid (up to 60%), whereas solvents like n-butanol–acetic acid–water (4 : 1 : 5) move them all near to the solvent front. Other basic systems, such as n-butanol–water–ammonia (sp. gr. 0·880) (85 : 12 : 3) and isopropanol–water–ammonia (sp. gr. 0·880) (100 : 20 : 10), give good spreads of compact spots, but the separation of the isoflavones from one another was not as good as in aqueous ammonia. Besides diazotised sulphanilic acid, other sprays tried were diazotised *p*-nitroaniline,[13] tetrazotised benzidine,[14] silver nitrate,[15] 1% alcoholic ferric chloride, and 5% alcoholic aluminium chloride.[12] The colour reactions of the isoflavones with these reagents are summarised in Table II.

Table II

Colour reactions of isoflavones on paper

	u.v.	u.v. + NH_3	u.v. + $AlCl_3$	dSA	dNA	tB	1% $FeCl_3$	$AgNO_3$
Biochanin A	dk	dk	bY	Br	Br	R	P-Br	+
Genistein	dk	dk	bY	Br	Br	R	Br-P	+
Formononetin	lB	blB	—	—	—	—	—	—
Daidzein	lB	blB	—	R-Br	lBr	—	—	—

u.v. = ultra-violet light, long λ; dk = dark; Br = brown
dSA = diazotised sulphanilic acid; b = bright; R = red
dNA = diazotised *p*-nitroaniline; l = light; P = purple
tB = tetrazotised benzidine; B = blue; Y = yellow

With a combination of fluorescence in ultra-violet light (for formononetin, daidzein and coumestrol) and azo-dye formation with diazotised sulphanilic acid (for biochanin A and genistein), less than 10 μg. each of the oestrogenic constituents of red clover can be detected after chromatographic separation.

The ultra-violet absorption spectra of spots 1, 2 and 3 on the two-dimensional chromatogram (Fig. 1) are similar to those of genistein and biochanin A suggesting their identity as isoflavones. Their concentrations however are very low, similar to those of daidzein and genistein. The isolation and characterisation of these compounds have been reported since this paper was submitted.[12a]

Estimation of oestrogens

The levels of the isoflavones in fresh clover leaves as determined by spectrophotometric measurements are summarised in Table III. It was not possible to obtain quantitative recovery of the isoflavones from the chromatogram by elution with ethanol. In control experiments

Table III

Concentrations of oestrogens in red clover leaves (mg.-% fresh clover)

	Sample I	Sample II
Formononetin	>2·3	>1·6
Biochanin A	1·7	1·0
Genistein	2·4	3·3
Daidzein	0·6	1·8
Coumestrol	0	<0·1

with known amount of isoflavones, recoveries varied from 60 to 85% for the different compounds. Individual compounds however gave consistent percentage recoveries. The mean percentage recovery and standard deviation for each isoflavone obtained from seven determinations over the range 25–200 µg. are as follows: biochanin A, 68 ± 3·5; genistein, 63 ± 3·9; formononetin, 74 ± 5·6; daidzein, 82 ± 2·2%. The results in Table III have been corrected for incomplete elution.

The quantitative extraction of formononetin was impracticable because of its low solubility in the common solvents. In the extracting procedure used, it was noticed that substantial amounts of formononetin usually remained in the interphase as insoluble scums. The actual level of formononetin in the plant material was therefore higher than was determined here.

The level of coumestrol on the chromatograms was found to be far too low to measure by ultra-violet absorption, but its intense whitish violet fluorescence under ultra-violet light makes it possible to estimate it visually. As little as 0·1 µg. of coumestrol on the two-dimensional chromatograms (corresponding to a concentration of 0·02 mg.-% in the plant) was easily detectable under ultra-violet light. The intensity of the coumestrol spot from Sample II was of that order.

Quantitative estimation of the individual oestrogens in the stems and petioles was not made. Two-dimensional paper chromatography showed that the compositions of the leaf and stem fractions were very similar. The level of oestrogens in the stems and petioles was, however, much lower than that in the leaves.

Results from the present work clearly show that formononetin and biochanin A are the two major isoflavones of red clover. Together they constitute the major portion of the ether soluble fraction after removal of chlorophyll and lipid material. By comparison, genistein and daidzein are present only in very low concentrations. The approximate concentrations of the isoflavones and coumestrol in red clover meal have been estimated by Guggolz *et al.*[8] Their results for the isoflavones are similar in relative proportions to those given here but are much lower in magnitude, but their figure for coumestrol is much higher than that found here.

A detailed comparison of the oestrogenic activity of the four isoflavones (Wong & Flux, unpublished results*) has shown that genistein, biochanin A and daidzein are all approximately of the same order of activity, with a variation of only 2–3 fold, whereas formononetin appears to be inactive. Coumestrol has been reported to be approximately 30–40 times more active than the isoflavones,[14] but it is present here in such a minute amount as to be considered negligible.

In view of the relatively low concentrations of genistein and daidzein, and the inactivity of formononetin, the oestrogenic activity of red clover is therefore due predominantly to biochanin A. The activity of freeze-dried clover has been found to be equivalent to about 9–10 mg. biochanin A per g. of dry clover (Wong & Flux, unpublished results*), equivalent to a level of 150–170 mg.-% fresh weight. The values for biochanin A given here are in good agreement with the bioassays. In subterranean clover, the level of genistein has been estimated to be of the order of 4–7 mg. per g. of dry clover.[17] The concentration of biochanin A has also been found to be appreciable.[3] Thus it appears that genistein and biochanin A are the major oestrogens in subterranean clover, whereas, in red clover, biochanin A is the only important oestrogenic constituent.

Acknowledgment

The author wishes to thank Dr. C. R. Thompson of the U.S. Department of Agriculture, Albany, California, for the gift of a sample of coumestrol.

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Received 19 September, 1961; amended paper 16 October, 1961

* (Note added in proof.) To be published in *J. Endocrin.*

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* We are grateful to the authors for preliminary information on this paper.

NOTE ON DETECTION OF MALATHION IN FOODSTUFFS

By J. A. McFARLANE

Recent work at the A.R.C. Pest Infestation Laboratory (Slough) and at the Tropical Products Institute (London) has shown that standard methods for the determination of the insecticide malathion (*S*-1,2-dicthoxycarbonylethyl *OO*-dimethyl phosphorodithioate) may give erroneous results when used to estimate residues in certain foodstuffs. This difficulty can usually be overcome by appropriate modification of the method.

Some results obtained previously may therefore be affected. In particular, residues [illegible] quoted after surface application of a malathion water-dispersible insecticide powder to bagged rice-bran (McFarlane, J. A., *J. Sci. Fd Agric.*, 1961, 12, 675) should not be accepted without question. It should not be assumed that the results referred to are necessarily invalid, but [illegible] circumstances it must be observed that check tests should be made if the use of malathion for such purposes is contemplated.

Details of the work which has brought these problems to light will be given in a paper to be published by the workers concerned (Bates, A. N., Rowlands, D. G., and Harris, A. H.), and this note is submitted for prior publication with their approval. A further experiment to test the uptake of malathion by various commodities, including rice-bran, has been set up at the Pest Infestation Laboratory, and the results will be published as soon as possible.

c/o Scott Agricultural Laboratories
P.O. Box 30028, Nairobi
Kenya
(Present address: c/o Pest Infestation Laboratory, Slough)

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Biol. Chem., Vol. 33, No. 3, p. 391~397, 1969]

Chemical Studies on "Clover Sickness"

Part I. Isolation and Structural Elucidation of Two New Isoflavonoids in Red Clover

By Saburo TAMURA, Ching-Fun CHANG, Akinori SUZUKI and Sumio KUMAI*

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**National Institute of Animal Industry, Nishinasuno, Tochigi-ken*

Received August 16, 1968

Two new isoflavonoids were isolated from red clover as germination inhibitors for the same plant and their structures were determined as a glucoside of biochanin A (7-O-β-glucosyl-3,7-dihydroxy-4'-methoxyisoflavone) (II) and its 5-malonate (I), respectively. Besides these compounds the following substances were also isolated as inhibitors: trifolirhizin (III), ononin (IV), daidzein (V) and its 7-glucoside (VI), formononetin (VII), genistein (VIII) and biochanin A (IX).

Red clover (*Trifolium pratense* L.) has been well known to exhibit homogeneous allelopathy, so called "clover sickness". In the course of the research on the cause of this phenomenon, extracts of red clover were found to show considerable inhibitory action on the seed germination of the same plant.[1] Then, isolation of the active principles contained in the extracts was undertaken by the present authors, and the results have been preliminarily reported in the previous paper.[2] Here the isolation and structural elucidation of two new isoflavonoids together with other various compounds obtained as germination inhibitors will be described in details.

At first, leaves and stems of red clover were extracted with methanol. Then ethyl acetate-soluble acidic and neutral fractions were separated from the methanol extract and treated as illustrated in Fig. 1a. The former acidic fraction was purified by silicic acid partition chromatography and crystallized from aqueous methanol to give a biologically active principle (I) as colorless needles melting at 214~216°C. After evaporation of the solvent, the neutral fraction was applied to a charcoal column. As shown in Fig. 1b, successive elution with aqueous acetone, acetone and acetone-aqueous ammonia gave a new isoflavonoid (II) besides seven compounds which were identified as trifolirhizin[3] (III), ononin,[4] daidzein[5] (V) and its 7-glucoside[6] (VI), formononetin[7] (VII), genistein[8] (VIII) and biochanin A[9] (IX). The compound II was obtained from the eluate with 90% aqueous acetone as needles melting at 207~209°C. No inhibitor other than the nine compounds

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7) S. Siddiqui, *J. Sci. and Ind. Research (India)*, 4, 68 (1945).

mentioned above was isolated from red clover extracts in this experiment.

The molecular formula $C_{21}H_{21}O_{13}$ was assigned to the acidic substance I through elemental analysis and molecular weight determination. Its IR spectrum (Fig. 2) reveals the presence of polyhydroxy and three carbonyl bands together with the presence of an isoflavone nucleus.[10] On mild hydrolysis with 0.01 N methanolic hydrochloric acid, I gave a

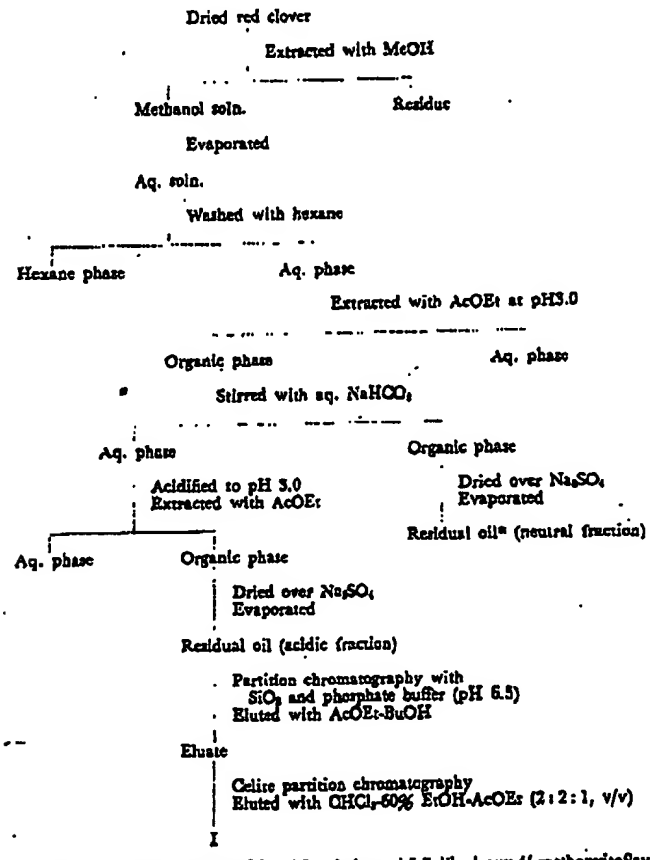

FIG. 1a. Isolation Procedure for 5-Malonyl-7-D-β-glucosyl-5,7-dihydroxy-4'-methoxyisoflavone (I).
* Treatment of this fraction is illustrated in Fig. 1b.

10) J. H. Looker and W. W. Hanneman, *J. Org. Chem.*, 27, 381 (1962).

H2O-CH3COCl

Trifoliriz

FIG. 1b. Is
Compou

1650

FIG. 2.

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King *et al.*,[11]
chanin A, 5

11) F. B.
J. Chem. Soc.,

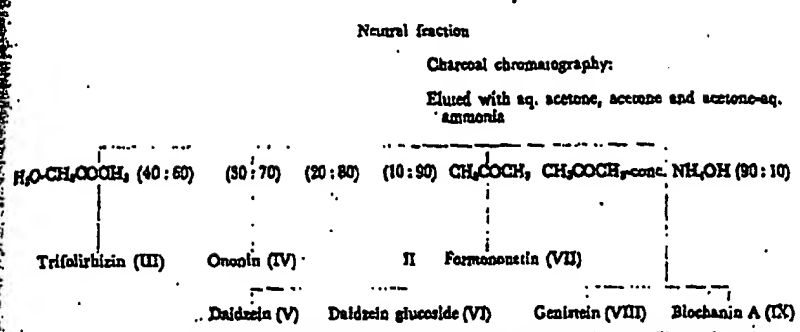

FIG. 1b. Isolation Procedure for 7-D-β-Glucosyl-5,7-dihydroxy-4'-methoxyisoflavone (II) and Other Compounds III-IX.

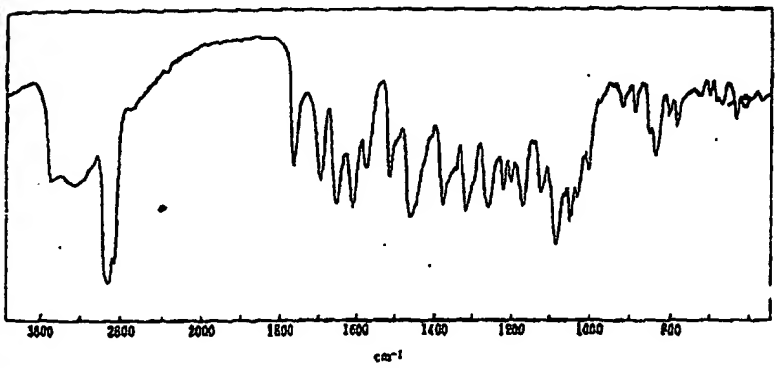

FIG. 2. IR Spectrum of 5-Malonyl-7-D-β-glucosyl-5,7-dihydroxy-4'-methoxyisoflavone (I) in Nujol.

neutral compound, $C_{28}H_{32}O_{15}$, completely identical with the compound II mentioned above.

When II was hydrolyzed with concentrated phosphoric acid, it afforded D-glucose and an isoflavone, and no other compound could be detected in the hydrolyzate. By comparison of UV and IR spectra as well as by chemical degradation according to the report of F. E. King *et al.*,[11] the isoflavone was identified as biochanin A, 5,7-dihydroxy-4'-methoxyisoflavone, which was first isolated from *Cicer arietinum* by S. Siddiqui.[8] Thus, II was confirmed to be a new glucoside consisting of each one mole of D-glucose and biochanin A.

In the UV spectrum of II (Fig. 3), the absorption maximum at 263 mμ in neutral ethanol solution did not shift in alkaline conditions.[12] This suggests that the glucose moiety is attached to the C-7 of the isoflavone nucleus. The location of the linkage was further confirmed by the inaction of II with

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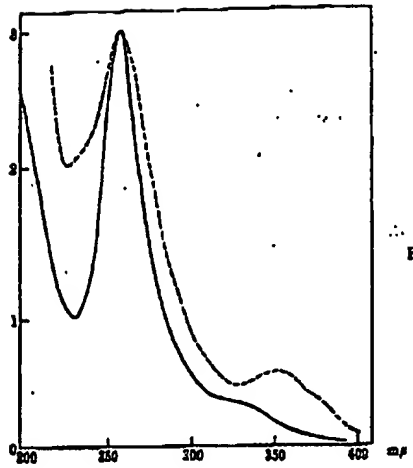


FIG. 3. UV Spectra of Biochanin A 7-glucoside (II).
—— in EtOH, —— in 0.01 N NaOH-EtOH.

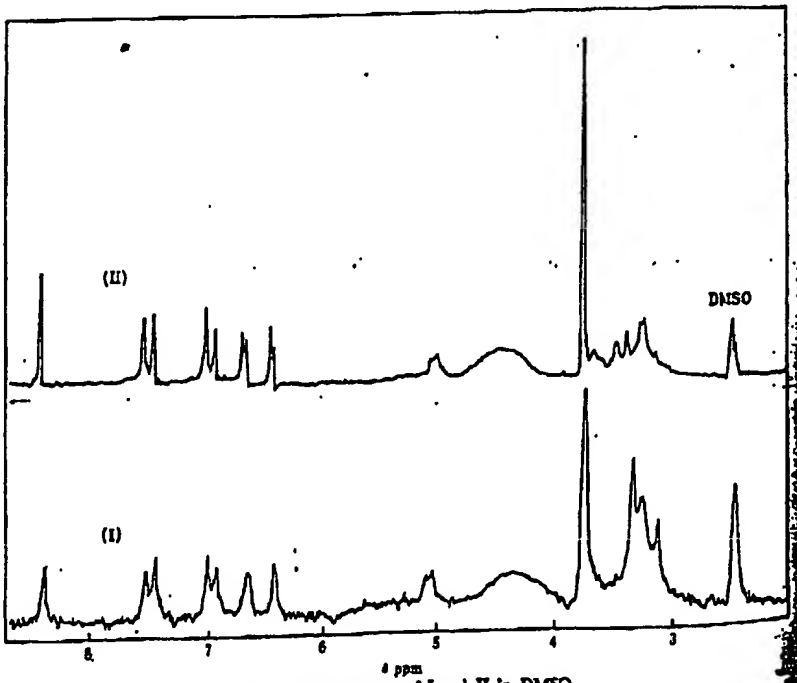
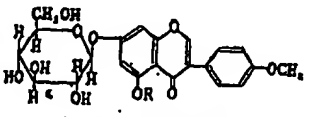


FIG. 4. NMR Spectra of I and II in DMSO.

diazomethane.[10] In the NMR spectrum of II (Fig. 4), a 1H doublet at δ 5.07[a] was assigned to the C-1''H of the glucose moiety, and the coupling constant, 7.5 cps between C-1''H and C-2''H indicated the β-configuration. Since the signal at δ 5.07 was not deshielded by acetylation of II, C-7 and C-1'' should be connected with one another. Thus, II has been established to be 7-*O*-β-glucosyl-5,7-dihydroxy-4'-methoxy-isoflavone. This may be the first example for the isolation of a glucoside of biochanin A.



(I) R-$COCH_2CO_2H$
(II) R-H

When I was mildly hydrolyzed with dilute methanolic hydrochloric acid as mentioned earlier, carbon dioxide and acetic acid were produced besides II. Moreover, a trace amount of dimethyl malonate was detected, when hydrolyzate was treated with diazomethane and subjected to gas chromatography as shown in Fig. 5. The $C_3H_2O_3$ difference in the molecular formulas of I and II corresponds to the presence of a malonyl moiety in the former. The bands at 1698 and 1768 cm^{-1} in the IR spectrum of I can be respectively assigned to the two carbonyls of the malonyl residue constituting respectively the free carboxyl and the ester bond with phenol. Further, a 2H singlet at δ 3.35 in the NMR spectrum of I (Fig. 4) should be assigned to the methylene group in the malonyl moiety. Thus, the structure of I has been established as 5-malonyl-7-*O*-β-glucosyl-5,7-dihydroxy-4'-methoxyisoflavone, which is a novel hemi-

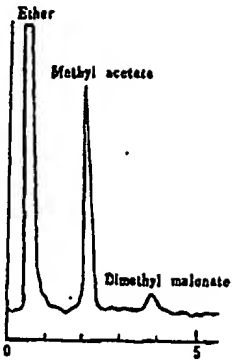


Fig. 5. Gas Chromatogram of Ether-Soluble Acidic Fraction Derived from Hydrolysis of I.

Column (3×225 mm): diethylene glycol succinate (Shimalite, 60~80 mesh, 15%); Column temp.: 120°C; Sample chamber temp.: 145°C; Detection temp.: 170°C; Carrier gas: N_2 30 ml/min.

malonate among the isoflavonoids isolated so far.

The compounds reported here showed inhibitory activity on germination of red clover seeds at the concentrations of 30 to 1000 ppm. The details of the biological activities will be reported elsewhere.

EXPERIMENTAL

IR measurements were made with a JASCO-S KCl spectrophotometer, and UV spectra were measured with a Cary-*PM* spectrophotometer. NMR spectra were measured with a JNM-4H-100 spectrometer. Gas chromatography was performed on a Shimadzu Gas Chromatograph GC-1B.

Isolation of an acidic isoflavonoid (I), 5-malonyl-7-O-β-glucosyl-5,7-dihydroxy-4'-methoxyisoflavone. As indicated in Fig. 1a, 50 kg of leaves and stems of red clover were dried in shade and extracted with three 50 l-portions of methanol. The combined extracts were evaporated under reduced pressure to 5 l of the aqueous residue, which was shaken with 4 l of hexane. The aqueous phase was adjusted to pH

10) T. A. Geissman and R. O. Clinton, *J. Am. Chem. Soc.*, 68, 697, 700 (1964).

a) Chemical shifts are expressed in δ-values (ppm) from tetramethylsilane as an internal standard.

3.0 with dilute hydrochloric acid and extracted with three 2.5 l-portions of ethyl acetate. The extracts were concentrated to 3 l under reduced pressure and stirred with three 2 l-portions of saturated aqueous sodium bicarbonate. The combined aqueous layers were acidified to pH 3.0 and extracted with three 3 l-portions of ethyl acetate. The extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure to yield 100 g of a residual syrup. Ten grams of the syrup was dissolved in 50 ml of ethyl acetate and applied to a column by using 150 g each of silicic acid (*Mallinckrodt*) and 0.1 N phosphate buffer (pH 6.5). The column was successively eluted with 2 l-portions of ethyl acetate containing 1.0, 2.0 or 3.0% butanol saturated with the above-mentioned phosphate buffer. The eluate with ethyl acetate containing 3% butanol afforded 1.5 g of an yellow oil, which was further applied to a column of 20 g of celite impregnated with the upper layer of the mixture of chloroform-60% ethanol-ethyl acetate (2 : 2 : 1, v/v). The column was successively eluted with 50 ml-portions of the lower layer of the above mentioned solvent system. Fractions No. 5~8 were combined and evaporated under reduced pressure to give 10 mg of I. Recrystallization from aqueous methanol afforded colorless needles, mp 214~216°C. *Anal.* Found: C, 56.07; H, 4.43. Calcd. for $C_{28}H_{26}O_{13}$: C, 56.39; H, 4.54%. UV λ_{max}^{EtOH} mμ(ε): 263 (29,000), 335 (3500). IR ν_{max}^{Nujol} cm^{-1}: 3200, 1768, 1698, 1660, 1615, 1580, 1518.

Isolation of a neutral isoflavonoid (II), 7-O-β-glucosyl-5,7-dihydroxy-4'-methoxyisoflavone. As indicated in Fig. 1b, the remaining ethyl acetate solution after separation of the acidic fraction by stirring with saturated aqueous sodium bicarbonate was evaporated under reduced pressure to give 150 g of an yellow syrup. Ten grams of the syrup was applied to a 100 g-charcoal column and the column was successively eluted with 2 l-portions of water containing acetone increased by ten percent steps. The eluate with 90% aqueous acetone was evaporated under reduced pressure to give 1.2 g of an yellow powder, which was further applied to a 15 g-charcoal column with the same manner as mentioned above. The fraction with 90% aqueous acetone was evaporated under reduced pressure to give 150 mg of crude II. Recrystallization from aqueous ethanol afforded colorless needles, mp 207~209°C. *Anal.* Found: C, 58.95; H, 4.82. Calcd. for $C_{28}H_{32}O_{15}$: C, 59.19, H, 4.97%. UV λ_{max}^{EtOH} mμ(ε): 253 (30,000), 336 (3000). IR ν_{max}^{Nujol} cm^{-1}: 3200, 1660, 1580, 1520, 1493.

Isolation and identification of compounds III-IX. As shown in Fig. 1b, trifoliirhizin (III) and ononin (IV) were obtained from the eluate with 60 and 70% aqueous acetone from the above mentioned charcoal column in yields of 45 and 210 mg, respectively. Further, 10 mg of daidzein (V), 2 mg of its 7-glucoside (VI), 350 mg of formononetin (VII), 15 mg of genistein (VIII) and 200 mg of biochanin A (IX) were obtained respectively.

III and VII were assigned by the IR and UV spectra and melting points which were identical with reported data for the compounds. Other five compounds were proved by comparisons of their melting points and chemical reactions with those in the earlier reports. Moreover, these assignments were supported by the following reactions. When daidzein glucoside (VI) was hydrolyzed with concentrated phosphoric acid, it gave free daidzein (V). Treatment of VI with diazomethane afforded ononin (IV). Further, hydrolysis of IV with concentrated phosphoric acid yielded formononetin (VII). When V and VII were treated with diazomethane, they afforded 4',7-dimethoxy-isoflavone. In a similar way genistein (VIII) and biochanin A (IX) gave 4',7-dimethoxy-5-hydroxy-isoflavone.

Mild acid hydrolysis of I

Formation of carbon dioxide—To 3 ml of methanol containing 0.05 ml of 1 N hydrochloric acid was added 50 mg of I, and the mixture was refluxed at 50°C for 30 min in a stream of nitrogen purified with acid, alkali and aqueous potassium permanganate successively. Carbon dioxide evolved was passed through 20 ml of aqueous solution containing 0.8 g of barium hydroxide and 0.4 g of barium chloride. The precipitated barium carbonate was separated, washed successively with water, 95% ethanol and ether and dried under reduced pressure. The yield was 17.3 mg which corresponds to 3.7 mg of carbon dioxide.

Formation of acetic and malonic acids—After the end of the hydrolysis, the reaction mixture was neutralized with dilute sodium hydroxide and the solvent was evaporated under reduced pressure. Then, water was added to the residue to amount to 50 ml, and the resulting solution was treated as follows:

a) One half of the solution was subjected to steam distillation, and 200 ml of the distillate was neutralized with 0.68 ml of 0.01 N sodium hydroxide which corresponds to 2.79 ml of acetic acid assuming that it

ould be the predominant organic acid. The remaining aqueous solution after steam distillation was used for the isolation of II.

(b) Another half of the solution was acidified with dilute hydrochloric acid and extracted with three 20 ml-portions of ether. The aqueous layer was used for the isolation of II. The combined ether extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was treated with 2 ml of ethereal diazomethane and the solvent was removed to yield an oily mixture of methyl esters, which was subjected to gas chromatography to reveal the presence of a minute amount of dimethyl malonate as shown in Fig. 5.

Formation of the isoflavonoid (II).—The above mentioned aqueous residues were combined and evaporated to a solid, which was then dissolved in 1 ml of ethanol. After separation of insoluble matter, the solution was evaporated under reduced pressure to give 40 mg of the isoflavonoid (II). Recrystallization from methanol afforded colorless needles, mp 207~209°C.

Hydrolysis of II and its identification as biochanin A glucoside. To a solution of 200 mg of II in 10 ml of 50% aqueous ethanol was added 1 g of concentrated phosphoric acid. The mixture was heated at 115°C for 3 hr in a sealed tube and then poured into 100 ml of ice-water to give crystals. Recrystallization from aqueous methanol afforded 110 mg of colorless needles, mp 212~214°C, which were identified as biochanin A based on the following data. *Anal.* Found: C, 67.72; H, 4.10. Calcd. for $C_{16}H_{12}O_5$: C, 67.60; H, 4.26%. UV λ_{max}^{EtOH} mμ(ε): 263 (30,000), $\lambda_{max}^{EtOH-NaOH}$ mμ(ε): 274 (30,000), 336 (9000). IR ν_{max}^{Nujol} cm^{-1}: 3475, 3380, 1660, 1631, 1579, 1532, 1500, 1258, 1190. By alkali fusion, the crystals afforded 2,4,6-trihydroxybenzoic acid and *p*-hydroxybenzoic acid. In addition, treatment with hot aqueous barium hydroxide gave anisic acid.

The filtrate, obtained after separation of biochanin A, was concentrated to 10 ml under reduced pressure and applied to a column of 25 ml of IR-120 in H form. The column was eluted with 100 ml of water, and the eluate was concentrated to 10 ml. This was further applied to a column of 25 ml of IR-45 in OH form and eluted with 100 ml of water. The eluate was evaporated under reduced pressure to afford 80.1 mg of a syrup, which was subjected to paper chromatography in different solvent systems such as phenol-water (95 : 5, v/v), collidine and butanol-acetic acid-water (4 : 1 : 5, v/v). In each case a spot corresponding to D-glucose was obtained. Further, phenylhydrazone of D-glucose obtained from the natural source melted at 206~208°C and gave no depression in the melting point mixed with an authentic sample. Thus, II was identified as biochanin A glucoside.

Pentaacetate of II. After treatment of 50 mg of II with 5 ml of pyridine and 3 ml of acetic anhydride at 100°C for 1 hr, the reaction mixture was poured into 50 ml of ice-water. The resulting crude crystals were dissolved in 20 ml of ethyl acetate, and the solution was washed with saturated aqueous sodium bicarbonate, dried over anhydrous sodium sulfate and evaporated under reduced pressure to give crystals. Recrystallization from ethyl acetate-hexane afforded 45 mg of pentaacetate of II. Mp 204~206°C. *Anal.* Found: C, 58.76; H, 4.92. Calcd. for $C_{26}H_{24}O_{10}$: C, 58.65; H, 4.91%. IR ν_{max}^{Nujol} cm^{-1}: 1778, 1752, 1293, 1258, 1223.

Acknowledgements. We wish to express our thanks to Mr. K. Aizawa of this Department for the measurements of IR, UV and NMR spectra. We are also grateful to The Central Laboratories of Sankyo Co., Ltd. for microanalysis.

J. Endocrin. (1962), 24, 341-348
Printed in Great Britain

THE OESTROGENIC ACTIVITY OF RED CLOVER ISOFLAVONES AND SOME OF THEIR DEGRADATION PRODUCTS

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(*Received* 28 *December* 1961)

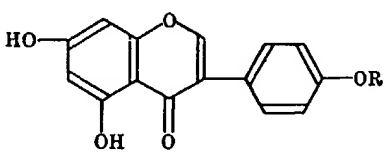
SUMMARY

The oestrogenic activities of the isoflavones biochanin A, genistein, formononetin and daidzein have been measured at three dose levels using the mouse uterine weight assay. The relative activities found were: genistein 1·5, biochanin A 1·0, daidzein 0·4. Formononetin had very little or no oestrogenic activity. The equivalent activity of a sample of Montgomery red clover, expressed in terms of biochanin A, was found to be in good agreement with that expected from concentrations of isoflavones determined from chemical analysis. A study was also made of *p*-hydroxyphenylacetic acid and the benzyl phenyl ketones related to the four isoflavones. These are products of chemical degradation of the isoflavones and were thought to be possible metabolic products of isoflavones in animals. None of these substances were found to have oestrogenic activity.

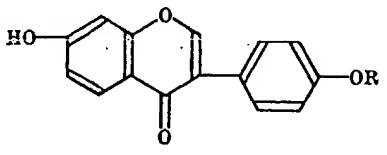
INTRODUCTION

Oestrogenic activities of isoflavones

The isoflavones biochanin A (I), genistein (II) and formononetin (III) are known to be present in red clover (*Trifolium pratense*) as well as in subterranean clover (*Trifolium subterraneum*) and are probably responsible for most of the oestrogenic activity of these pasture species (see reviews of Bradbury & White, 1954; Pope, 1954; Biggers, 1959). Another closely related isoflavone, daidzein (IV), previously found in soya bean as the glucoside, has also recently been detected in red clover and other forages (Guggolz, Livingston & Bickoff, 1961; Wong, 1962).



(I) R=CH_3
(II) R=H



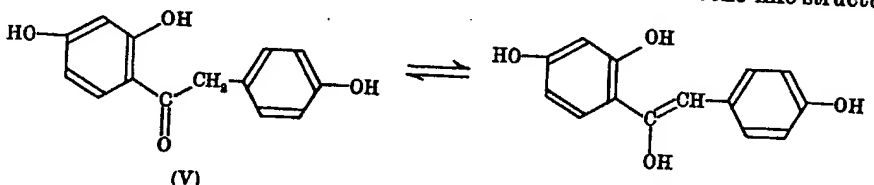
(III) R=CH_3
(IV) R=H

The oestrogenic activities of these isoflavones have been compared with that of diethylstilboestrol by Cheng, Yoder, Story & Burroughs (1954). They found daidzein to be somewhat more active than genistein and biochanin A, and formononetin to have a slight activity. In contrast to these results, Bradbury & White (1954), Pope (1954), and Biggers & Curnow (1954) have found formononetin to be inactive in mice at a variety of dose levels. Bradbury & White (1954) also found daidzein to have no oestrogenic effect in mice at a dose of 5·4 mg. given by injection. Pope (1954) reported that the oestrogenic activity of biochanin A was approx. 0·63 that of genistein.

In order to evaluate the contributions of these constituents to the total activity of red clover, more accurate estimates of their oestrogenic activities are necessary. Also in view of the findings that formononetin and biochanin A are the predominant isoflavones in red clover (Guggolz *et al.* 1961; Wong, 1962) the question of the activity, if any, of formononetin becomes of importance. In this paper the results of a comparative study of the activity of the isoflavones at three dose levels using the mouse uterine weight bioassay are presented, together with the results of a comparison of the oestrogenic activity of a sample of red clover and biochanin A.

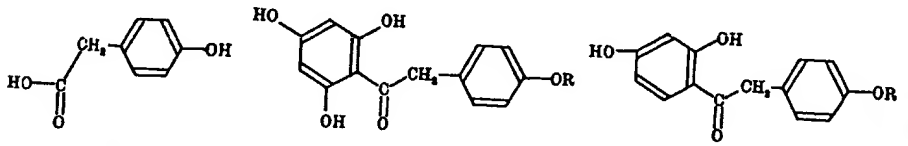
Possible metabolic products of isoflavones in animals

Biggers & Curnow (1954) have reported that genistein behaved as a pro-oestrogen, the oestrogenic activity following its administration probably being the property of a metabolite. The ultimate metabolic fate of isoflavones in animals is unknown. Chemically isoflavones are easily degraded under alkaline conditions to benzyl phenyl ketones (e.g. (V)). These under more drastic conditions can be broken down further to phenols and phenylacetic acids. It seemed possible that isoflavones could be similarly degraded in the animal and that some of these compounds could be oestrogenic. The benzyl phenyl ketones could conceivably enolise to give rise to stilbene-like structures



(V) (VI)

(e.g. (V) ⇌ (VI)) which could be oestrogenic. The increase in oestrogenic activity of plant materials (Beck & Curnow, quoted by Curnow, 1954) and of genistein (Pieterse & Andrew, 1956) after alkaline treatments may have been due to the formation of such compounds. The oestrogenic activities of *p*-hydroxyphenylacetic acid (VII) and the ketones (VIII)–(XI) corresponding to the isoflavones (I)–(IV), respectively, have now been studied.



(VII)

(VIII) R=CH_3
(IX) R=H

(X) R=CH_3
(XI) R=H

MATERIALS

p-Hydroxyphenylacetic acid (VII)

This acid was prepared from *p*-methoxyphenylacetonitrile which was obtained from *p*-methoxybenzaldehyde via the cyanhydrin by the method of Kindler & Peschke (1933). The nitrile (1 g.) and conc. hydriodic acid (S.G. 1·7, 15 ml.) were refluxed for 4 hr., the reaction mixture diluted with water (2 vol.), treated with sodium metabisulphite and the resulting yellow solution extracted with equal volumes of ether (×3). The ether soluble residue was recrystallized twice in 5 ml. water (charcoal) to give *p*-hydroxyphenylacetic acid (360 mg.), colourless needles, m.p. 153–4° C (Found: C, 63·3; H, 5·2; O, 31·7. Calc. for $C_8H_8O_3$: C, 63·1; H, 5·3; O, 31·5%).

4-Methoxybenzyl 2,4,6-trihydroxyphenyl ketone (VIII)

This substance was obtained from phloroglucinol and *p*-methoxyphenylacetonitrile by the Hoesch reaction, according to Badcock, Cavill, Robertson & Whalley (1950). It crystallized as needles from aqueous alcohol, m.p. 197° C.

4-Methoxybenzyl 2,4-dihydroxyphenyl ketone (X)

This was prepared according to Baker & Eastwood (1929) from resorcinol and *p*-methoxyphenylacetonitrile by the Hoesch reaction. It crystallized as needles from aqueous alcohol, m.p. 159° C (Found: C, 69·1; H, 5·2; OCH_3, 12·1. Calc. for $C_{14}H_{11}O_3$–(OCH_3): C, 69·8; H, 5·5; OCH_3, 12·0%).

4-Hydroxybenzyl 2,4,6-trihydroxyphenyl ketone (IX)

4-Methoxybenzyl 2,4,6-trihydroxyphenyl ketone (VIII) from above (1 g.) was refluxed with aluminium chloride (10 g.) in benzene (100 ml.) for 4 hr. with stirring. Benzene was removed under reduced pressure and ice-cold water (50 ml.) added to the residue followed by an equal volume of 6 N-HCl. The resulting suspension was extracted three times with 100 ml. portions of ether. The ether extract was evaporated and the residue taken up in boiling water and filtered. The filtrate on cooling deposited small needles, indefinite m.p. 246–51° C. Paper chromatography in the solvent system benzene-acetic acid-water (125:72:3) showed that the product contained unchanged starting material. Cellulose column partition chromatography with the above solvent system resulted in the separation of the two components. The product was recrystallized in water, yielding small needles m.p. 260°C (253–7°; Walz, 1931).

Attempts to demethylate (VIII) to (IX) with hydriodic acid (S.G. 1·7) resulted in the formation of phloroglucinol and *p*-hydroxyphenylacetic acid.

4-Hydroxybenzyl 2,4-dihydroxyphenyl ketone (XI)

This was obtained from 4-methoxybenzyl 2,4-dihydroxyphenyl ketone (X) by demethylation with aluminium chloride in benzene as described for (IX) above. The product was purified by chromatography through the same partition column. It crystallized from water as fine needles, m.p. 191–2° C in agreement with that reported by Walz (1931). Treatment of (X) with hydriodic acid resulted in resorcinol and *p*-hydroxyphenylacetic acid.

Biochanin A (I)

This was synthesized from 4-methoxybenzyl 2,4,6-trihydroxyphenyl ketone (VIII) and ethoxalyl chloride by the method of Baker, Chadderton, Harborne & Ollis (1953). Recrystallization from aqueous alcohol yielded needles, m.p. 214° C. Acetate m.p. 191–2° C.

Formononetin (III)

This isoflavone was obtained from 4-methoxybenzyl 2,4-dihydroxyphenyl ketone (X) by the ethyl orthoformate-pyridine method of Sathe & Venkataraman (1949). It crystallized as plates from alcohol, m.p. 256–7° C. Acetate m.p. 171° C.

Daidzein (IV)

Formononetin (800 mg.) was refluxed with hydriodic acid (S.G. 1·7, 15 ml.) for 3½ hr. The mixture after cooling overnight deposited a light-brown precipitate which was filtered off. The dark mother liquor was diluted with 3 vol. water whereby further material was precipitated. The mixture was treated with sodium metabisulphite and filtered. The combined precipitates were recrystallized in alcohol (*ca.* 75 ml.) to yield an off-white powder (520 mg.). Further crystallization in alcohol yielded needles, m.p. 324° C (decomp.). Acetate m.p. 187° C.

Genistein (II)

This was obtained similarly by demethylation of biochanin A, with conc. hydriodic acid (cf. Shriner & Hull, 1945). It crystallized as needles from alcohol, m.p. 296° C (decomp.). Acetate m.p. 202° C.

BIOASSAYS

The mice were from the same colony and were treated in the same way as those used by Munford & Flux (1961). Ovariectomized females 21–23 days of age were allotted at random to treatment groups, six being used at each dose level of each material to be tested.

For all experiments a meal consisting of one part dried buttermilk powder to two parts wholemeal flour was used. Materials to be tested were added to this as follows.

(i) For the bioassay comparisons of oestrogenic activities of pure compounds, these were ground finely and mixed with meal. This was fed at the rate of 2·5 g./mouse/day for 6 days. The quantities of isoflavones added would give total doses of 3, 6 and 12 mg./mouse, respectively, to the mice on the three dose levels. For diethylstilboestrol the total doses were arranged to be 0·036, 0·072 and 0·144 μg./mouse.

(ii) For the comparison of the oestrogenic activity of biochanin A and red clover leaves and petioles, powdered ryegrass, known to be free from oestrogenic activity, was added to the meal containing red clover powder or biochanin A so as to standardize the plant-material content of all the diets at 10 %. Red clover was added to the meal to give three total dose levels, planned as 375, 750 and 1500 mg./mouse, respectively, and biochanin A was added to give total dose levels of 5, 10 and 20 mg./mouse. The meal containing these materials was fed in the same way as that in (i).

(iii) For the tests for oestrogenic activity the compounds were added to the meal in the same way as those described in (i) above except that the only dose level used was 12 mg./mouse. These mixtures were also fed at a rate of 2·5 g./mouse/day for 6 days.

The criterion used as an indication of the presence of oestrogenic activity was a statistically significant increase in the mean uterine weight of the treated mice over that of control mice. Transformation of data was not necessary in the tests for oestrogenic activity because the uterine weights of the mice differed little within or between groups and the actual weights could be analysed statistically. In the bioassays, however, where three dose levels of each compound or clover were used and uterine weights were affected by the treatments, the variance increased with the means, making transformation of uterine weights to logarithms necessary. The relationship of $\log_{10}$ dose and $\log_{10}$ uterine weight in each case was linear or close to linear. Relative potencies were calculated according to the methods described by Finney (1950).

RESULTS

Comparison between genistein, biochanin A, daidzein, formononetin and diethylstilboestrol

The results of the experiment in which genistein, biochanin A, formononetin and diethylstilboestrol were compared are shown in Table 1 and those for biochanin A and daidzein in Table 2. The dose rates planned were achieved for diethylstilboestrol and a close approximation was obtained for formononetin, but a falling off of intake as dose of isoflavone increased was seen with genistein and biochanin A.

Table 1. *Uterine and vaginal responses to isoflavones and diethylstilboestrol, and relative potencies of biochanin A, genistein and diethylstilboestrol*

Material	Total dose /mouse (six mice/group)	Mean uterine weight (mg.)	No. of positive vaginal smears
Controls	—	5·9 ± 0·13*	0
Formononetin	3·00 mg.	6·1 ± 0·13	0
	5·99 mg.	6·4 ± 0·13	0
	11·96 mg.	6·7 ± 0·13	0
Genistein	2·99 mg.	6·8	0
	5·77 mg.	12·3	5
	11·13 mg.	23·3	6
Biochanin A	2·94 mg.	7·0	0
	5·90 mg.	8·2	4
	11·72 mg.	14·2	5
Diethylstilboestrol	0·036 μg.	7·9	0
	0·072 μg.	9·7	0
	0·144 μg.	16·0	0

Relative activities based on uterine weight response (95% fiducial limits): genistein/biochanin A, 1·49 (0·97–2·29); genistein/diethylstilboestrol, $13{\cdot}5 \times 10^{-6}$ ($10{\cdot}3$–$17{\cdot}8 \times 10^{-6}$); biochanin A/diethylstilboestrol, $9{\cdot}1 \times 10^{-6}$ ($6{\cdot}0$–$13{\cdot}7 \times 10^{-6}$).

* S.E.M.'s apply to control and formononetin treated mice only.

Formononetin did not cause significant increases in uterine weight, nor did any of the mice to which it was given show vaginal opening. Genistein and biochanin A caused increase in uterine weight, and, at the higher dose rates, caused most of the mice to show positive vaginal smears. Diethylstilboestrol caused increased uterine weights, but, at the dose levels used, no vaginal responses. The bioassays (Table 1) showed that genistein was the most potent of the isoflavones and biochanin A was

about two thirds as active as genistein. No valid bioassay was possible with formononetin because its dose response line differed too much in slope from the others. Both genistein and biochanin A were much less active than diethylstilboestrol, the ratio of the activities being of the order of 1:100,000.

The activity of daidzein was about two-fifths of that of biochanin A (Table 2) and at the highest dose rate used it caused only one mouse to show a positive vaginal smear although all six showed vaginal opening. In this respect it differed little from the dose of biochanin A which had a similar effect on uterine weight.

Table 2. *Uterine and vaginal responses to daidzein and biochanin A, and potency of daidzein relative to biochanin A*

Material	Total dose/mouse (mg.; six mice/group)	Mean uterine weight (mg.)	No. of positive vaginal smears
Daidzein	3·0	5·8	0
	6·0	7·7	0
	12·0	7·9	1
Biochanin A	3·0	7·7	0
	6·0	13·6	4
	12·0	14·4	5

Activity of daidzein relative to biochanin A, 0·39 (95% fiducial limits, 0·25–0·44).

Table 3. *Uterine and vaginal responses to red clover and biochanin A, and estimated potency of red clover in terms of biochanin A*

Material	Total dose/mouse (mg.; six mice/group)	Mean uterine weight (mg.)	No. of positive vaginal smears
Biochanin A	4·85	6·2	0
	9·20	15·1	5
	18·60	26·6	6
Red clover (leaf and petiole)	360	6·5	1
	670	10·6	1
	1400	17·6	5

Activity of red clover leaves and petioles in terms of biochanin A, 10·1 mg./g. dry clover (95% fiducial limits, 7·6–13·2).

Table 4. *Uterine and vaginal responses to some isoflavone degradation products*

Material*	Mean uterine weight (mg.)	No. of positive vaginal smears
(A) Controls	6·6 ± 0·13	0
4-Hydroxybenzyl 2,4,6-trihydroxyphenyl ketone	6·7 ± 0·13	0
4-Hydroxybenzyl 2,4-dihydroxyphenyl ketone	7·1 ± 0·13	0
p-Hydroxyphenylacetic acid	6·6 ± 0·13	0
(B) Controls	5·1 ± 0·18	0
4-Methoxybenzyl 2,4,6-trihydroxyphenyl ketone	4·7 ± 0·18	0
4-Methoxybenzyl 2,4-dihydroxyphenyl ketone	5·5 ± 0·18	0

* Total dose for each 12 mg./mouse.

Equivalent activity of red clover

The results of the estimation of the oestrogenic activity of a sample of red clover leaves and petioles in terms of biochanin A are shown in Table 3. The activity of the clover was estimated as equivalent to that of 10·1 mg. biochanin A/g. dry clover. Positive vaginal smears were recorded for most mice fed at the highest dose rate.

Oestrogenic activity of degradation products

The results of the tests for oestrogenic activity of *p*-hydroxyphenylacetic acid and the benzyl phenyl ketones corresponding with genistein, daidzein, biochanin A and formononetin are shown in the two parts of Table 4. None caused uterine weights to increase significantly over those of the control mice in the same experiment, nor did they cause vaginal opening.

DISCUSSION

In the present study formononetin was found to have little or no oestrogenic effect when fed to mice in total doses of up to 12 mg./mouse, this result being in accordance with those of Bradbury & White (1954), Pope (1954) and Biggers & Curnow (1954) with similar doses, given by injection. Cheng *et al.* (1954) who used only one dose level, 10 mg./mouse given orally, reported a small increase in the mean uterine weight of treated mice over that of controls. However, the weight of the evidence suggests that formononetin has little or no oestrogenic effect when given by injection or orally to mice in doses of the order of 12 mg. or less.

The estimates of the relative activities of genistein, biochanin A and daidzein of 1·5:1·0:0·4, respectively, differ from those of Cheng *et al.* (1954) who reported daidzein to be more active than genistein or biochanin A. However, those in the present study, being based on more adequate bioassays, are to be preferred. The relative activity of genistein and biochanin A found was in close agreement with that of Pope (1954).

The 95% fiducial limits for the activity of genistein in terms of biochanin A included the value of one, but as this was just within the lower limit it is unlikely that genistein was not the more active compound. The upper 95% fiducial limit for the estimate of the activity of daidzein relative to biochanin A was only 0·44, hence there was little doubt that daidzein was the less active compound in this bioassay.

The estimated activity of genistein in terms of diethylstilboestrol in the present study was about 50% higher than that obtained earlier in the same laboratory (Munford & Flux, 1961) but included the lower estimate within its fiducial limits.

The quantal information of the frequencies of positive vaginal smears was not used for bioassays because of the small numbers of animals involved but served to confirm that the isoflavones genistein and biochanin A were oestrogens or gave rise to oestrogens. The single positive vaginal smear for daidzein was insufficient support for any conclusion on this compound. As was the case with the earlier comparison of the activities of diethylstilboestrol and genistein by Munford & Flux (1961), it appears that the estimated relative activities of the oestrogenic isoflavones and diethylstilboestrol might be different if the occurrence of positive vaginal smears in mice was used instead of increase in uterine weight.

The activity of the red clover sample found here, equivalent to a concentration of

10·1 mg. biochanin A/g. dry clover (95% fiducial limits, 7·6–13·2 mg./g.) is in good agreement with concentration of isoflavones as determined from chemical analysis (Wong, 1962). Results for two samples of the same New Zealand Montgomery red clover were 8·4 and 9·4 mg. biochanin A/g. dry clover. The concentration of formononetin was also found to be high, but genistein and daidzein were negligible by comparison. These results from bio- and chemical assays show that the oestrogenic activity of red clover could be mainly accounted for by biochanin A. They also confirm that formononetin contributes little if anything to the oestrogenic activity of the plant material.

The inactivity of the ketones and *p*-hydroxyphenylacetic acid shows that these are not the active metabolites of isoflavones. Pieterse & Andrew (1956) have reported that when genistein was degraded by refluxing for 3 hr. with 2·5% alcoholic potassium hydroxide and the resulting solution fed to mice, about a fourfold increase in oestrogenic activity was found. Curnow (1954) has also quoted evidence that treatment of subterranean clover 'chloroplast' with alkali yields a small amount of a substance with oestrogenic activity at least ten times that of genistein. In view of the inactivity of the *p*-hydroxyphenylacetic acid and the benzyl phenyl ketones which would be expected to be the major products formed after alkaline treatment of isoflavones, the increase in activity found by these workers must be due to some minor products.

The oestrogenic activities discussed here apply to mice. The activities of the isoflavones in ruminants are not known. Nilsson (1961) has shown that transformation of biochanin A to genistein occurs in rumen fluid *in vitro*. If this demethylation can take place with formononetin to give daidzein, and if daidzein is active in ruminants, then the presence of large amounts of formononetin in red clover, although not important with mice, may be significant oestrogenically in sheep.

The tenure of a research grant from the Department of Scientific and Industrial Research (N.Z.) by one of us (D.S.F.) is gratefully acknowledged.

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0005648023 CAB Accession Number: 19851475727

Contents of different constituents of important fodder plants. 5. Carotene, phytosterol and isoflavone contents.

Original Title: Untersuchungen zu den Gehalten an verschiedenen

Inhaltsstoffen wichtiger Futterpflanzen. 5. Gehalte an Carotin,

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Wirtschaftseigene Futter vol. 30 (3): p.184-201

Publication Year: 1984

ISSN: 0049-7711

Language: German Summary Language: English Record Type:

Abstract

Zeitschrift „Das wirtschaftseigene Futter", Band 30, Heft 3, Seite 184 – 201

Hessische Lehr- und Forschungsanstalt für Grünlandwirtschaft und Futterbau
– Eichhof – Bad Hersfeld

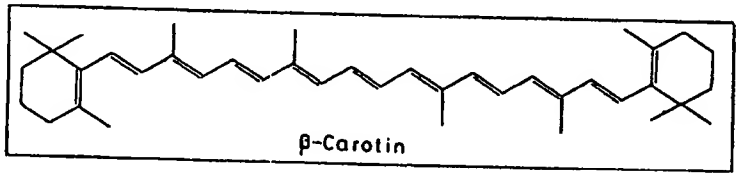
Untersuchungen zu den Gehalten an verschiedenen Inhaltsstoffen wichtiger Futterpflanzen*

5. Mitteilung: Gehalte an Carotin, Phytosterinen und Isoflavonen

D. Puffe, F. Morgner und W. Zerr

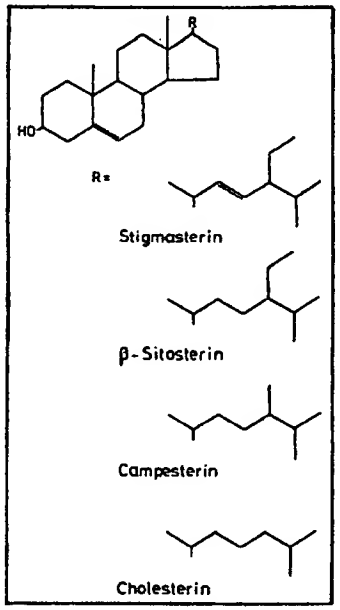
1. Einleitung

Die vorliegende Mitteilung mit Ergebnissen von Untersuchungen an einigen Futterpflanzenarten bildet den Abschluß vorangehender Arbeiten (PUFFE, MORGNER u. ZERR, 1984, 1. – 4. Mitt.). Das eingangs abgehandelte Carotin kommt in allen grünen Pflanzenteilen vor und ist dort in den Chloroplasten als Begleiter des Chlorophylls anzutreffen. Bei rotgelber Färbung führt es zu einer gewissen Lichtabsorption und somit auch zu einer möglichen Wirkung bei der Photosynthese (MENGEL, 1979). Wegen seiner Bedeutung als Vorstufe zum Vitamin A, in welches es im tierischen Organismus übergeht, muß es bei den landwirtschaftlichen Nutztieren mit dem Futter in ausreichender Menge zugeführt werden (PAPENDICK, 1955, 1956; TIEWS, 1969). Ausgangsprodukt zur Bildung von Carotin in der Pflanze ist das Acetyl-Coenzym A, welches in direkter Verbindung zum Kohlenhydratstoffwechsel steht. Nach Bildung von Mevalonsäure und schließlich Isoprenbausteinen als Zwischenprodukten kommt es zur Polymerisation und schrittweisen Entstehung von Carotin, welches in die Gruppe der Tetraterpene einzuordnen ist. Unter verschiedenen Isomeren kommt die Hauptbedeutung dem β-Carotin zu (GROB, 1963; GOODWIN, 1965; NUHN, 1981).



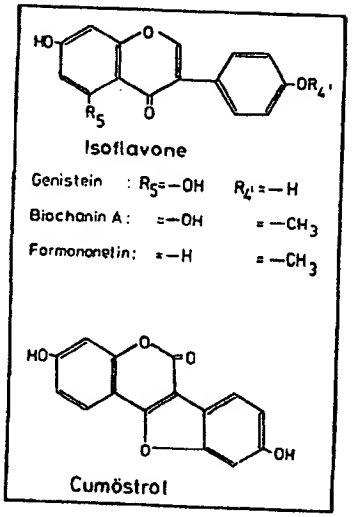
Ebenfalls vom Acetyl-Coenzym A ausgehend und wiederum über die Mevalonsäure sowie über die Bildung von Isoprenbausteinen führt durch Polymerisation der Weg zum Triterpen Squalen, durch dessen Cyclisierung die Entstehung von carbocyclischen Verbindungen mit drei Sechser- und einem Fünferring ermöglicht wird. Dies sind die Phytosterine, welche wegen des Vorhandenseins von einer OH-Gruppe auch Phytosterole genannt werden.

*Die Untersuchungen wurden dankenswerterweise durch Mittel der Deutschen Forschungsgemeinschaft ermöglicht.



Das Cholesterin, welches in der Pflanze in geringer Menge nachgewiesen werden kann, ist vermutlich Ausgangsprodukt für andere Phytosterine, von denen eine große Anzahl bekannt ist. Hauptvertreter der Phytosterine sind das Sitosterin (besonders das β-Sitosterin), das Stigmasterin und das Campesterin. Diese unterscheiden sich untereinander wie auch vom Cholesterin lediglich geringfügig in der Seitenkette. In der Pflanze kommen die Phytosterine frei, als Glycoside oder auch als Ester vor (BERGMANN, 1953; HEUSNER, 1958; GOAD, 1967; HARBORNE, 1971 a; GOAD and GOODWIN, 1966, 1972; BEAN, 1973; KARLSON, 1974; GRUNWALD, 1975; SCHÜTTE, 1975; MENGEL, 1979; NUHN, 1981). Zu erwähnen ist noch, daß das Cholesterin neben anderen Stoffen eine Rolle bei der Bildung von Membranen in der Pflanzenzelle spielt (BEAN, 1973; GRUNWALD, 1975; MENGEL, 1979). Über weitere Funktionen der Phytosterine in der Pflanze ist wenig bekannt. Die nahe Verwandtschaft der Phytosterine zu den D-Vitaminen und zu den Sexualhormonen im tierischen Organismus ist besonders hervorzuheben. Für letztere ist das im tierischen Organismus in größerer Menge vorkommende Cholesterin die Ausgangssubstanz (KARLSON, 1974). Verschiedentlich wird vermerkt, daß Phytosterine im tierischen Organismus eine östrogene Wirkung hervorrufen (KARG u. VOGT, 1969; GRUNERT u. LOTTHAMMER, 1970, SCHULTZ, 1970), doch scheint es darüber noch keine genaueren Anhaltspunkte zu geben.

Wie bereits früher erwähnt (PUFFE, MORGNER u. ZERR, 1984, 1. u. 4. Mitt.) liegt der Ursprung aller „sekundären Pflanzenstoffe" phenolischer Art bei der an den Kohlenhydratstoffwechsel anschließenden Shikimisäure. Zwischen dieser und dem Phenylalanin kommt es zur Ausbildung eines Benzolrings. Auf dem Weg über die Zimtsäure und die p-Cumarsäure ist bei den Chalconen ein zweiter Benzolring angelegt, wodurch zu den eigentlichen Flavonoiden übergeleitet wird (GRISEBACH u. BARZ, 1963, 1964; SCHÜTTE, 1974; HAHLBROCK, 1981). Von den Flavonoiden interessieren in diesem Zusammenhang allerdings nur die Isoflavone und das Cumöstrol. Besonders wichtige Isoflavone sind Genistein, Biochanin A und Formononetin. Das Cumöstrol gehört nicht direkt zu den Isoflavonen, wenn auch zu diesen in der Struktur weitgehende Ähnlichkeit besteht und ein gleicher biogenetischer Ursprung zu verzeichnen ist (GRISEBACH u. BARZ, 1964). Doch ergibt sich beim Cumöstrol ein wesentlicher Unterschied durch einen Ringschluß im Molekül über eine O-Brücke. Isoflavone wie auch Cumöstrol treten in der Pflanze vornehmlich als Glycoside auf. Isoflavone und Cumöstrol kommen allerdings im wesentlichen nur bei Leguminosenarten in Betracht. In



dieser Hinsicht liegen von Kleearten und Luzerne wegen ihrer besonderen Bedeutung als Futterpflanzen zahlreiche Untersuchungsergebnisse aus verschiedenen Ländern vor. Die Untersuchungen gehen auf die Beobachtung zurück, daß Isoflavone und Cumöstrol durch östrogene Wirkung Fruchtbarkeitsstörungen, beobachtet bei Schafen und Rindern, hervorrufen können. Dies tritt ein, wenn es durch einseitige Fütterung zu einer Überdosierung der genannten Stoffe kommt. Die ersten Berichte hierzu betrafen vor etwa vierzig Jahren Trifolium subterraneum (Bodenfrüchtiger Klee) (BENNETTS, 1944; HANSON et al., 1965; BICKOFF et al., 1962; SCHULTZ, 1965, 1966, 1967 a und b, 1970; GRUNERT, WOELKE u. SCHULTZ, 1967; KARG u. VOGT, 1969; STÄHLIN, 1969; GRUNERT u. LOTTHAMMER, 1970; HARBORNE, 1971 b; WONG, 1975; GOSDEN, 1978). Bei der Prüfung der Aktivität im biologischen Test an Mäusen und Ratten wurde für das in der Struktur verwandte Sexualhormon Östron ein Wert von 6900 gefunden, während Werte von 35 für das Cumöstrol, 1,0 für das Genistein, 0,46 für das Biochanin A und 0,26 für das Formononetin ermittelt wurden (BICKOFF et al., 1962; KARG u. VOGT, 1969). Nach JARITZ, 1980, ist von den drei Isoflavonen Genistein, Biochanin und Formononetin das zuletzt genannte am stärksten östrogen aktiv, wobei in Trifolium subterraneum Gehalte ab 8000 mg/kg TS als hoch gelten.

2. Versuchsdurchführung

Zur Bestimmung der Gehalte an Carotin (PAPENDICK, 1960) wurde frisches Pflanzenmaterial mittels Ultra-Turrax methanolisch extrahiert, der Extrakt mit Benzin ausgeschüttelt und eine säulenchromatographische Abtrennung des Carotins über Aluminiumoxid vorgenommen. Gemessen wurde kolorimetrisch bei 470 nm. Die Bestimmung der Phytosterine wurde wie bei anderen Autoren (GRUNWALD, 1970; PATTERSON, 1971; HOMBERG, 1977; PAILER u. RIEDL, 1978) gaschromatographisch durchgeführt. Frisches Pflanzenmaterial wurde am Rückfluß mit Alkohol extrahiert, der Extrakt eingeengt, eine Verseifung vorgenommen, der Alkohol abdestilliert und der Rückstand in Diethylether aufgenommen. Danach erfolgte eine dünnschichtchromatographische Trennung auf Kieselgel mit Cyclohexan, Essigsäureethylester und Ethanol (9/9/2) als Laufmittelgemisch. Die Sterinfraktion wurde von der Dünnschichtplatte eluiert, mit Bis(trimethylsilyl)-acetamid (BSA) derivatisiert und gaschromatographisch unter Verwendung einer Glassäule mit 2 % OV 17 auf Chromosorb getrennt.

Zur Bestimmung der Isoflavone liegen verschiedene methodische Arbeiten vor (SCHULTZ, 1965; SCHULTZ, 1967 b; RITTER, 1976). Bei dem eigenen, etwas modifizierten Verfahren (ZERR, 1980) wurde frisches Pflanzenmaterial zur Spaltung der glycosidischen Bindung der Isoflavone und des Cumöstrols durch pflanzeneigene Enzyme bebrütet und danach mit Alkohol erschöpfend extrahiert. Der Extrakt wurde am Rotationsverdampfer eingeengt und auf eine Kieselgur-Fertigsäule gegeben. Mittels Petrolether wurden die lipoiden Anteile entfernt und danach die flavonoide Fraktion mittels Diethylether eluiert. Das Eluat wurde eingeengt und eine dünnschichtchromatographische Trennung auf Kieselgel mit einem Laufmittelgemisch aus Chloroform und Methanol (9/1) vorgenommen. Die einzelnen Stoffe wurden unter der UV-Lampe lokalisiert und mit Methanol von der Platte eluiert. Die photometrische Messung erfolgte für Genistein sowie Biochanin A bei 263, für Formononetin bei 250 und für Cumöstrol bei 245 nm.

Für die verschiedenen Futterpflanzenarten werden in den Abbildungen 2 bis 4 die folgenden Abkürzungen benutzt: Lieschgras Li, Knaulgras Kn, Wiesenschwingel WS, Deutsches Weidelgras DW, Welsches Weidelgras WW, Persischer Klee PK, Rotklee RK, Weißklee WK, Luzerne Lu.

3. Versuchsergebnisse

3.1 Gehalte an Carotin

Die in Abbildung 1 dargestellten Ergebnisse aus dem Versuchsjahr 1979 zeigen bei den Grasarten, besonders im Welschen Weidelgras und Deutschen Weidelgras, hohe Anfangsgehalte und eine starke Abnahme der Gehalte mit fortschreitendem Entwick-

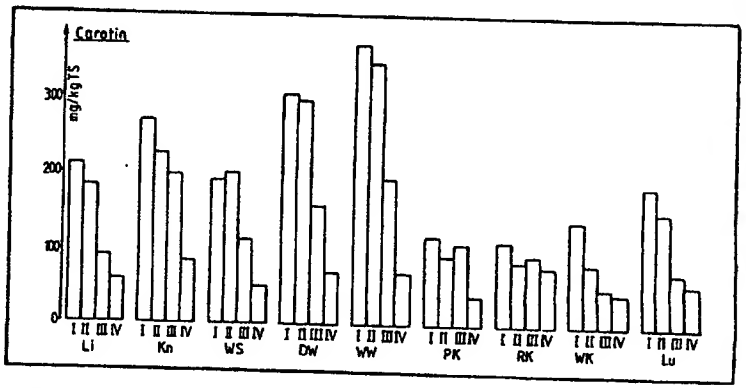


Abbildung 1: Gehalte an Carotin (gesamte Pflanze*) in mg/kg Trockensubstanz bei fünf Gras- und vier Leguminosenarten (Schnittzeiten I – IV/1979)
* „gesamte Pflanze" = gesamte oberirdische Pflanzenteile

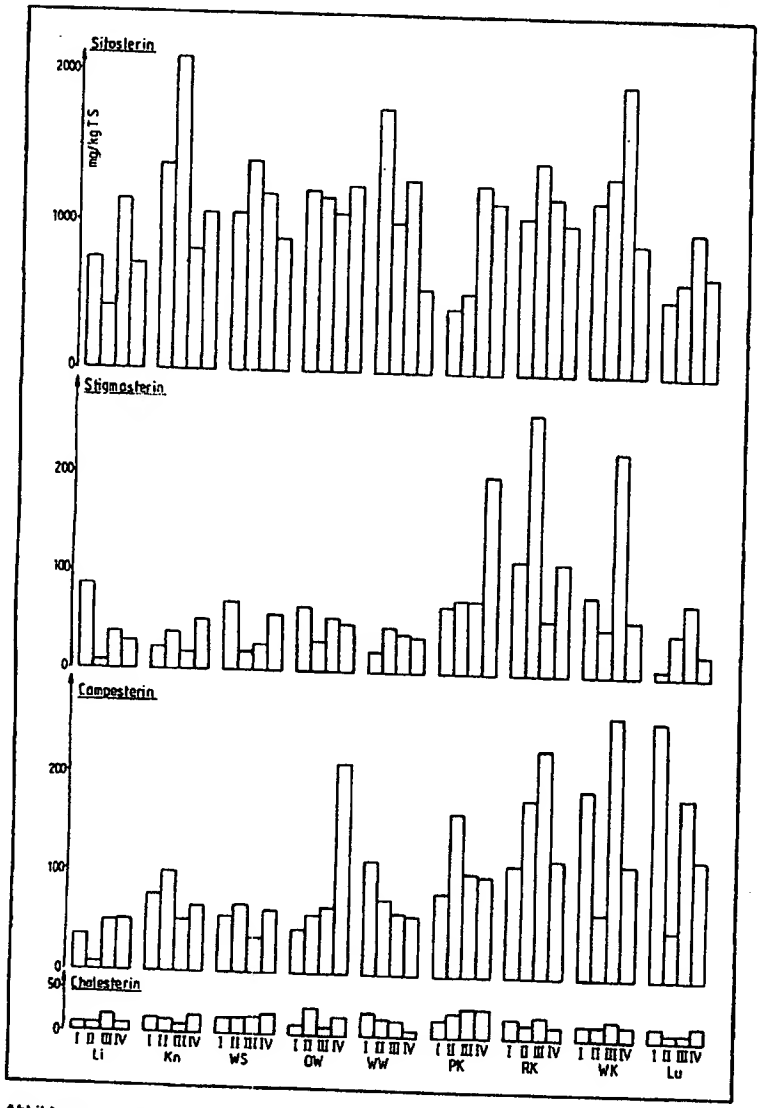


Abbildung 2: Gehalte an Phytosterinen (gesamte Pflanze) in mg/kg Trockensubstanz bei fünf Gras- und vier Leguminosenarten (Schnittzeiten I – IV/1979)

lungsstadium. Bei den vier Leguminosenarten ergeben sich geringere Anfangsgehalte, jedoch anschließend ebenso ein deutlicher Abfall.

3.2 Gehalte an Phytosterinen

Nach den Ergebnissen in Abbildung 2 aus dem Versuchsjahr 1979 tritt bei den Phytosterinen das Sitosterin am stärksten hervor, wobei der unterschiedliche Maßstab in der Abbildung zu beachten ist. Mit großem Abstand folgen das Stigmasterin und das Campesterin, während ein nur geringer Anteil vom Cholesterin eingenommen wird. Mit Ausnahme von zeitweilig erhöhten Gehalten an Stigmasterin bei den drei Kleearten sind keine Besonderheiten bestimmter Arten sichtbar. Auch hinsichtlich der Veränderungen mit fortschreitendem Entwicklungsstadium ist keine deutliche Tendenz zu erkennen.

Tabelle 1: Gehalte an Phytosterinen (gesamte Pflanze bzw. Pflanzenteile) in mg/kg Trockensubstanz bei Lieschgras und Knaulgras (Schnittzeiten I – IV/1980)

Schnittzeit	I	II	III	IV	I	II	III	IV
	mg Sitosterin/kg TS				mg Stigmasterin/kg TS			
	Lieschgras				Lieschgras			
Gesamt	691,7	693,0			6,0	16,0		
Blatt			1921,8	621,9			66,5	28,4
Stengel			480,3	197,0			13,2	16,2
Blütenstände				998,7				101,1
	Knaulgras				Knaulgras			
Gesamt	1456,9	914,8			17,3	17,2		
Blatt			805,5	550,2			29,0	23,3
Stengel			239,0	159,6			11,1	18,4
Blütenstände				568,1				45,7
	mg Campesterin/kg TS				mg Cholesterin/kg TS			
	Lieschgras				Lieschgras			
Gesamt	22,5	23,3			15,6	9,3		
Blatt			53,4	20,4			21,0	12,5
Stengel			20,0	11,0			15,1	7,7
Blütenstände				77,4				12,4
	Knaulgras				Knaulgras			
Gesamt	67,9	43,8			19,8	15,9		
Blatt			31,2	29,9			19,7	5,4
Stengel			33,3	30,6			17,9	13,0
Blütenstände				46,1				5,4

Tabelle 2: Gehalte an Sitosterin und Stigmasterin (ges. Pflanze) in mg/kg Trockensubstanz bei verschiedenen Sorten von Knaulgras, Deutschem Weidelgras, Welschem Weidelgras und Rotklee (Versuchsjahr 1981)

	Mähweideschnitt 1.	2.	3.	4.	Siloschnitt 1.	2.	
	mg Sitosterin/kg TS						
Oberweihst	621,1	385,5	332,2	779,0	352,9	673,7	
Holstenkamp	704,0	292,7	628,7	1066,1	452,3	623,3	Knaulgras
Baraula	953,5	504,0	1389,9	588,3	679,5	731,8	
NFG	210,9	945,4	272,3	1175,5	475,1		
Gremie	651,1	367,1	433,2	483,1	161,4		
Verna	584,8	527,6	1298,8	566,7	145,6		Deutsches Weidelgras
Barpastra	134,1	481,9	303,6	742,1	873,5		
Vigor	377,1	741,8	683,3	510,8	759,0		
Lema	142,2	316,1	157,1	746,0			
Lemtal	316,7	446,2	298,8	49,1			
Nachwuchsfroh	845,3	835,2	206,3	149,7			Welsches Weidelgras
Meritra	396,9	429,5	934,7	197,7			
(Zuchtstamm)	303,6	409,3	459,8	369,1			
Tapiopoly	418,3	313,8	170,8	425,0			
Lucrum	338,7	411,8	626,2	847,1			
Remy	276,2	459,6	531,6	293,3			Rotklee
Oberhaunstädter	220,1	689,8	386,6	790,3			
	mg Stigmasterin/kg TS						
Oberweihst	31,2	16,5	11,3	23,9	24,5	36,8	
Holstenkamp	17,9	18,8	28,1	52,8	46,0	34,8	Knaulgras
Baraula	26,5	15,1	50,2	33,6	27,3	58,3	
NFG	15,3	53,9	23,1	15,5	26,5		
Gremie	36,5	60,7	48,3	24,3	23,6		
Verna	45,4	91,0	97,1	39,4	25,0		Deutsches Weidelgras
Barpastra	26,5	69,7	25,3	27,6	45,1		
Vigor	27,5	62,6	27,8	67,6	40,3		
Lema	24,9	36,1	10,8	112,7			
Lemtal	21,3	36,4	44,5	15,4			
Nachwuchsfroh	70,6	66,6	15,0	8,0			Welsches Weidelgras
Meritra	24,9	14,2	71,9	15,7			
(Zuchtstamm)	20,2	21,9	37,1	31,2			
Tapiopoly	17,7	26,6	11,6	19,8			
Lucrum	40,1	29,3	17,2	54,1			
Remy	30,2	38,9	28,7	24,9			Rotklee
Oberhaunstädter	21,7	93,0	25,4	58,8			

Tabelle 3: Gehalte an Campesterin und Cholesterin (gesamte Pflanze) in mg/kg Trockensubstanz bei verschiedenen Sorten von Knaulgras, Deutschem Weidelgras, Welschem Weidelgras und Rotklee (Versuchsjahr 1981)

	Mähweideschnitt 1.	2.	3.	4.	Siloschnitt 1.	2.	
	mg Campesterin/kg TS						
Oberweihst	9,9	10,7	3,1	7,7	9,7	8,3	
Holstenkamp	5,5	5,6	18,0	9,9	15,8	8,5	Knaulgras
Baraula	7,9	3,7	7,6	16,4	7,1	10,3	
NFG	3,3	14,0	16,3	23,6	18,5		
Gremie	19,4	16,7	19,6	14,1	10,9		
Verna	14,8	25,0	74,3	28,9	10,9		Deutsches Weidelgras
Barpastra	3,4	13,5	16,5	18,8	13,6		
Vigor	24,0	42,8	36,8	22,1	28,0		
Lema	7,1	14,7	6,1	40,3			
Lemtal	10,5	14,6	27,1	8,9			
Nachwuchsfroh	28,6	38,0	13,6	6,7			Welsches Weidelgrsa
Meritra	9,7	7,0	22,1	7,8			
(Zuchtstamm)	9,4	8,2	34,1	12,9			
Tapiopoly	9,1	13,2	8,6	10,4			
Lucrum	15,7	13,6	6,1	20,9			
Remy	5,5	13,6	18,5	9,5			Rotklee
Oberhaunstädter	5,1	23,4	11,2	18,7			
	mg Cholesterin/kg TS						
Oberweihst	17,8	3,7	7,7	4,7	2,4	3,1	
Holstenkamp	10,4	30,2	8,7	11,9	14,6	11,9	Knaulgras
Baraula	5,7	5,8	5,1	8,4	6,8	20,6	
NFG	4,1	8,9	13,2	7,7	30,8		
Gremie	16,8	13,1	10,0	16,7	16,0		
Verna	6,3	13,3	28,5	33,6	16,0		Deutsches Weidelgras
Barpastra	3,0	19,0	21,0	19,7	30,3		
Vigor	34,8	23,6	25,2	14,9	14,5		
Lema	7,2	12,3	15,7	11,7			
Lemtal	7,4	9,0	42,3	14,0			
Nachwuchsfroh	11,3	12,6	15,8	5,2			Welsches Weidelgras
Meritra	6,5	12,6	17,2	12,4			
(Zuchtstamm)	10,3	15,8	17,3	5,3			
Tapiopoly	2,9	3,1	3,1	3,7			
Lucrum	17,8	7,0	5,9	6,7			
Remy	6,2	7,6	6,3	4,4			Rotklee
Oberhaunstädter	5,8	26,4	5,2	2,9			

Im Versuchsjahr 1980 sind bei zwei untersuchten Grasarten (Tab. 1) höhere Gehalte an Sitosterin und Stigmasterin im Blatt im Vergleich zu den Gehalten im Stengel anzutreffen, während relativ hohe Gehalte in den Blütenständen auftreten. Keine so klaren Unterschiede zwischen Blatt und Stengel liegen beim Campesterin und Cholesterin vor. In den Blütenständen sind relativ hohe Gehalte an Campesterin zu verzeichnen.

Für das Versuchsjahr 1981 mit mehreren aufeinanderfolgenden Schnitten (Tab. 2 u. 3) ergeben Untersuchungen an Rotklee und drei Grasarten keine typischen Unterschiede zwischen diesen Arten, allerdings gewisse Sortenunterschiede. Auch bei diesen Ergebnissen betragen die Gehalte an Sitosterin ein Mehrfaches der Gehalte an Stigmasterin und Campesterin, während die Gehalte an Cholesterin am geringsten ausfallen.

3.3 Gehalte an Isoflavonen

Nach den in Abbildung 3 dargestellten Ergebnissen tritt in den Versuchsjahren 1978/79 im Weißklee kein Genistein auf, während Persischer Klee, Rotklee und Luzerne nicht immer Gehalte in meßbarer Größenordnung aufweisen. Biochanin kommt in den genannten Jahren im Weißklee und in der Luzerne nicht vor und ist im Persischen Klee nur 1979 nachzuweisen. Durchweg relativ hohe Gehalte an Biochanin sind im Rotklee vorhanden. Hinsichtlich der Gehalte an Formononetin steht der Rotklee weitaus an der Spitze. Für Persischen Klee und Weißklee sind deutlich geringere Werte festzustellen, während die Gehalte an Formononetin in der Luzerne am geringsten sind. Die Cumöstrol-Gehalte der vier Leguminosenarten zeigen keine bedeutsamen Unterschiede. – Bei allen hier bestimmten Stoffen ist kein deutlicher Zusammenhang zwischen den Gehalten und dem jeweiligen Entwicklungsstadium zu erkennen.

Nach den Ergebnissen in den Tabellen 4 und 5 aus dem Versuchsjahr 1980 sind im Blatt an Genistein, Biochanin, Formononetin bzw. Cumöstrol meist höhere Gehalte zu finden als im Stengel. Sehr unterschiedliche Gehalte weisen die Blütenstände auf. Im Gegensatz zu den Vorjahren ist im Versuchsjahr 1980 Biochanin auch in der Luzerne nachzuweisen. Im Weißklee kommen das Biochanin und das in den Vorjahren ebenfalls fehlende Genistein hinzu.

Im Versuchsjahr 1981 (Tab. 6) sind die Gehalte an Biochanin im Rotklee wiederum relativ hoch. Genistein kommt diesmal im Rotklee in allen Fällen vor. Diese beiden Isoflavone sind im Weißklee und in der Luzerne nicht generell festzustellen. Hinsichtlich der Gehalte an Formononetin übertrifft der Rotklee die anderen Leguminosenarten wiederum beträchtlich. Bei den Gehalten an Cumöstrol ergeben sich auch hier keine charakteristischen Unterschiede zwischen den Arten, wohl aber gewisse Sortenunterschiede. – Während derartige Stoffe für Leguminosenarten typisch sind, kommen sie in Grasarten nicht vor.

4. Diskussion

Hinsichtlich der Carotingehalte im frühen Entwicklungsstadium ergibt sich nur zum Teil eine Übereinstimmung mit anderen Hinweisen (PAPENDICK, 1956, 1968; STÄH-

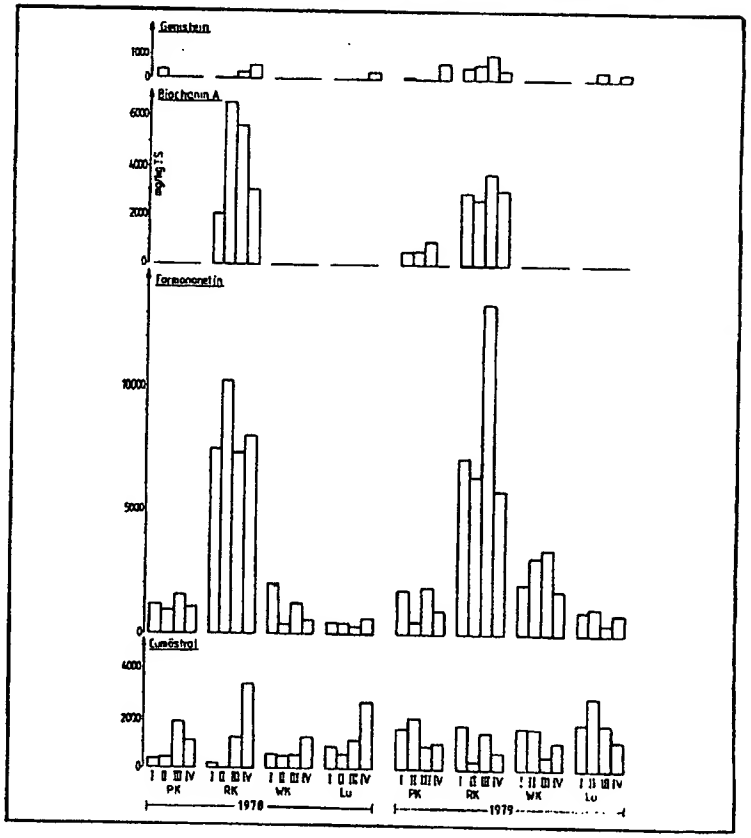


Abbildung 3: Gehalte an Genistein, Biochanin A, Formononetin und Cumöstrol (gesamte Pflanze) in mg/kg Trockensubstanz bei vier Leguminosenarten (Schnittzeiten I – IV/1978 – 1979)

LIN, 1969; TIEWS, 1969). Besonders bei den Leguminosenarten erscheinen die Werte der eigenen Untersuchungen relativ niedrig. Dies mag mit dem jeweils vorliegenden Verhältnis zwischen Blatt und Stengel und auch anderen Einflüssen (wie Düngung, Witterung usw.) zusammenhängen. Daß die Carotingehalte mit fortschreitendem Entwicklungsstadium abfallen, ist wohl die Regel und wird auf einen zunehmenden Stengelanteil zurückgeführt (PAPENDICK, 1956; TIEWS, 1969).

Von den vier bestimmten Phytosterinen ist auch nach anderen Angaben (PAILER u. RIEDL, 1978) der Anteil von Sitosterin in verschiedenen Grasarten sehr hoch. Für die

Tabelle 4: Gehalte an Genistein und Biochanin A (gesamte Pflanze bzw. Pflanzenteile) in mg/kg Trockensubstanz bei vier Leguminosenarten (Schnittzeiten I – IV/1980)

	mg Genistein/kg TS				mg Biochanin A/kg TS			
Schnittzeit	I	II	III	IV	I	II	III	IV
	Persischer Klee[1]				Persischer Klee[1]			
Gesamt	96,8	93,3			0	0		
Blatt	0	28,8			0	0		
Stengel		232,6				0		
Blütenstände								
	Persischer Klee[2]				Persischer Klee[2]			
Gesamt	88,5	332,9			140,8	0		
Blatt	0	0			0	0		
Stengel		273,1				0		
Blütenstände								
	Persischer Klee[3]				Persischer Klee[3]			
Gesamt	83,8	535,5			64,8	123,4		
Blatt	89,4	0			0	0		
Stengel		531,8				61,3		
Blütenstände								
	Rotklee				Rotklee			
Gesamt	535,7	0	106,7	37,0	2130,5	685,3	1112,2	1567,5
Blatt	0	32,9	0	22,0	220,8	140,1	146,5	507,3
Stengel			0	0			697,9	608,1
Blütenstände								
	Weißklee				Weißklee			
Gesamt	65,2	110,7	591,2	425,2	1410,7	0	2279,4	1917,5
Blatt	36,6	35,3	0	86,8	472,8	72,3	106,7	993,5
Stengel			218,0	0			971,4	2111,3
Blütenstände								
	Luzerne				Luzerne			
Gesamt	0	0	0	100,4	42,1	96,2	7,9	72,6
Blatt	0	0	45,3	52,1	109,6	0	30,8	67,5
Stengel			0	0			116,9	71,1
Blütenstände								

[1] Iranisches Handelssaatgut
[2] „Lupers"
[3] „Asterix"

Tabelle 5: Gehalte an Formononetin und Cumöstrol (gesamte Pflanze bzw. Pflanzenteile) in mg/kg Trockensubstanz bei vier Leguminosenarten (Schnittzeiten I – IV/1980)

	mg Formononetin/kg TS				mg Cumöstrol/kg TS			
Schnittzeit	I	II	III	IV	I	II	III	IV
	Persischer Klee[1]				Persischer Klee[1]			
Gesamt	599,0	651,6			161,3	218,7		
Blatt	247,2	217,8			48,5	390,3		
Stengel		295,7				425,9		
Blütenstände								
	Persischer Klee[2]				Persischer Klee[2]			
Gesamt	534,4	679,0			110,6	282,0		
Blatt	348,7	600,3			33,2	311,0		
Stengel		539,8				22,9		
Blütenstände								
	Persischer Klee[3]				Persischer Klee[3]			
Gesamt	947,7	687,7			105,5	442,5		
Blatt	219,0	271,7			316,8	281,7		
Stengel		326,5				430,3		
Blütenstände								
	Rotklee				Rotklee			
Gesamt	4621,2	2307,9	2616,2	2246,6	366,1	75,6	552,5	202,9
Blatt	286,9	886,3	755,1	642,1	138,5	32,9	217,8	147,4
Stengel			832,9	652,1			185,7	0
Blütenstände								
	Weißklee				Weißklee			
Gesamt	1386,7	446,9	710,5	674,5	364,4	247,4	1022,4	514,4
Blatt	736,4	337,8	135,2	469,8	328,7	260,8	313,5	335,2
Stengel			232,9	804,8			185,4	88,7
Blütenstände								
	Luzerne				Luzerne			
Gesamt	175,5	242,8	211,5	118,6	881,4	515,5	104,1	200,8
Blatt	91,4	41,7	221,6	249,1	717,9	390,2	439,4	345,8
Stengel			338,1	213,3			348,1	218,9
Blütenstände								

[1] Iranisches Handelssaatgut
[2] „Lupers"
[3] „Asterix"

Tabelle 6: Gehalte an Genistein, Biochanin A, Formononetin und Cumöstrol (gesamte Pflanze) in mg/kg Trockensubstanz bei verschiedenen Sorten von vier Leguminosenarten (Versuchsjahr 1981)

	Mähweideschnitt 1.	2.	3.	4.	1.	2.	3.	4.
	mg Genistein/kg TS				mg Biochanin A/kg TS			
	Persischer Klee				Persischer Klee			
Iran Asterix	439,4 176,2	241,4 137,9	0 271,6	100,7	282,9 229,2	306,3 145,4	365,4 0	134,9
	Rotklee				Rotklee			
Tapiopoly	536,4	522,6	704,2		2580,3	2352,3	3318,8	
Lucrum	557,3	492,6	1025,1		2774,9	1944,0	2063,6	
Remy	417,3	568,7	772,9		2005,3	3524,4	3211,5	
Oberhaunstädter	719,6	611,8	612,3		1572,0	3716,7	1003,9	
	Weißklee				Weißklee			
NFG Gigant	0	0	0		315,1	0	136,2	
Milka	339,0	281,6	0		0	195,1	0	
Milkanova	0	200,5	153,7		0	0	0	
	Luzerne				Luzerne			
Luna	0	0	0		0	201,5	362,7	
Franken neu	0	152,2	0		142,6	125,7	257,2	
Europe	0	0	0		171,2	0	213,4	
Langmeiler	0	0	0		120,5	0	0	
Warotte	290,0	0	0		108,8	0	147,6	
	mg Formononetin/kg TS				mg Cumöstrol/kg TS			
	Persischer Klee				Persischer Klee			
Iran Asterix	800,7 877,1	969,9 374,5	939,2 927,7	841,6	615,5 363,5	761,5 159,4	720,6 472,7	190,8
	Rotklee				Rotklee			
Tapiopoly	6573,7	5842,3	4823,0		439,9	744,6	463,0	
Lucrum	6618,2	4607,2	3915,6		537,9	546,4	1017,7	
Remy	3312,2	4608,8	4315,2		287,6	489,0	395,4	
Oberhaunstädter	3766,8	5168,0	2214,5		631,9	440,0	514,0	
	Weißklee				Weißklee			
NFG Gigant	1257,8	1562,0	401,6		664,8	940,9	570,3	
Milka	850,1	953,0	600,9		677,5	880,6	412,2	
Milkanova	1211,8	1239,6	1576,2		777,8	1085,9	770,4	
	Luzerne				Luzerne			
Luna	480,5	276,8	533,0		452,7	586,9	778,9	
Franken neu	231,5	386,6	351,6		363,2	711,0	524,2	
Europe	383,5	400,7	278,8		685,2	525,5	548,9	
Langmeiler	299,7	431,6	391,3		275,6	785,3	690,1	
Warotte	500,7	588,1	274,0		513,7	1291,4	455,0	

untersuchten Leguminosenarten liegen zwar Hinweise in der Literatur vor, jedoch keine quantitativen Angaben. Im übrigen steht, wie schon angedeutet, hinsichtlich der Funktion der Phytosterine in Pflanze und Tier noch ein umfangreicher Fragenkomplex offen, der u.a. die genetischen Beziehungen zu den D-Vitaminen und den Sexualhormonen umfaßt.

Bei den Isoflavonen werden verschiedentlich Gehalte an Genistein gefunden, welche über 20000 mg/kg TS liegen (SCHULTZ, 1967 b). Dies betrifft den hier nicht untersuchten Bodenfrüchtigen Klee (Trifolium subterraneum), bei dem allerdings erhebliche Sortenunterschiede zu verzeichnen sind. Für diese Kleeart werden außerdem Gehalte an Biochanin bis über 9000 mg/kg TS und Gehalte an Formononetin bis nahezu 9000 mg/kg TS genannt. Möglicherweise ist das gleichzeitige Vorkommen von hohen Gehalten dieser drei Isoflavone hinsichtlich der östrogenen Wirkung von Bedeutung. Aus zahlreichen Untersuchungen an Rotklee (GOSDEN u. JONES, 1971, 1977, 1979; GOSDEN, 1978; JONES, 1978; GOSDEN, DAVIES u. JONES, 1979) ergeben sich Gehalte an Formononetin bis ca. 20000 mg/kg TS, Gehalte an Biochanin bis ca. 9000 mg/kg TS und nur geringe Gehalte an Genistein. Bei ebenfalls geringen Gehalten an Genistein werden bei den eigenen Untersuchungen wesentlich geringere Gehalte an Formononetin und Biochanin im Rotklee erreicht. Zu den Gehalten an Genistein, Biochanin und Formononetin im Persischen Klee gibt es bisher keine Hinweise und auch zu den Gehalten im Weißklee kaum Angaben. Nach den vorliegenden Ergebnissen sind die Gehalte an diesen Isoflavonen in beiden Kleearten relativ gering. Insgesamt gesehen noch geringere Gehalte weist die Luzerne auf. Letztere wird gelegentlich als reich an dem nicht direkt zu den Isoflavonen gehörigen Cumöstrol bezeichnet. Doch zeigen die eigenen Untersuchungen keine charakteristischen Unterschiede zwischen den untersuchten Leguminosenarten. In älteren Literaturangaben (HANSON et al., 1965) werden allerdings meist wesentlich niedrigere Werte für Cumöstrol in der Luzerne angegeben. Es kann nichts darüber ausgesagt werden, inwieweit die hier vorgefundenen Gehalte an Cumöstrol bei der hohen östrogenen Aktivität dieses Stoffes (BICKOFF et al., 1962; KARG u. VOGT, 1969) eine Wirkung im Tier zeigen. – Daß die Isoflavone wie auch das Cumöstrol besonders im Blatt und weniger im Stengel vorkommen, wird allgemein bestätigt (HANSON et al., 1965; SCHULTZ 1965, 1966; GOSDEN u. JONES, 1979). Die beim Vergleich der Versuchsjahre auftretenden Unterschiede in den Gehalten sind vermutlich u.a. auf Witterungseinflüsse zurückzuführen. Von einem Anstieg der Gehalte in bestimmten Entwicklungsstadien, wozu es in den eigenen Untersuchungen keine Anhaltspunkte gibt, wird verschiedentlich berichtet (HANSON et al., 1965; JONES, 1978; GOSDEN u. JONES, 1979). – Es erscheint noch besonders bemerkenswert, daß diese in Leguminosenarten vorkommenden Stoffe mengenmäßig alle bei den vorliegenden Arbeiten abgehandelten sekundären Pflanzenstoffe weit übertreffen. Vielleicht besteht ein Zusammenhang mit dem Befund, daß die Vorläufer für die Isoflavone und das Cumöstrol im pflanzlichen Stoffwechsel, die Hydroxyzimtsäuren, in den Leguminosenarten gewöhnlich im geringeren Maße auftreten als in den Grasarten.

Berichtigung zu den Abbildung 1 und 2 (3. Mitteilung):

Die Dimension mit mg/kg T.S. ist durch % i.T.S. zu ersetzen.

5. Zusammenfassung

Die Untersuchungen betreffen fünf Grasarten und vier Leguminosenarten. Es ergeben sich deutlich abnehmende Gehalte an Carotin mit fortschreitendem Entwicklungsstadium.

Von den bestimmten Phytosterinen tritt besonders das Sitosterin hervor. Die Gehalte an Stigmasterin und Campesterin sind wesentlich niedriger, die Gehalte an Cholesterin relativ gering. Es zeigen sich Unterschiede in den verschiedenen Pflanzenteilen.

Das Isoflavon Genistein ist in allen untersuchten Leguminosenarten in nur geringen Mengen vertreten. Relativ hohe Gehalte an Biochanin und besonders an Formononetin treten im Rotklee auf, während diese beiden Isoflavone im Persischen Klee, im Weißklee und in der Luzerne weit zurücktreten. Hinsichtlich der Gehalte an Cumöstrol sind zwischen den verschiedenen Arten keine charakteristischen Unterschiede festzustellen. Die genannten Isoflavone bzw. das Cumöstrol sind hauptsächlich im Blatt lokalisiert. – In Grasarten kommen derartige Stoffe nicht vor.

Summary

Investigations on the contents of different plant substances in important forage plants

5. Carotin, phytosterols and isoflavones contents

Five grasses and four legumes were investigated. Decreasing contents of carotin were found with advanced development.

Of the phytosterols investigated sitosterol is of special importance. The contents of stigmasterol and campesterol are much lower and the contents of cholesterol relatively low. Differences between the parts of the plants were found.

The contents of the isoflavone genistein are relatively low in all legumes investigated. The contents of biochanin and formononetin are relatively high in red clover, but low in Persian clover, white clover and lucerne. Differences in contents of coumestrol between the species are unimportant. The isoflavones mentioned respectively coumestrol are mainly localized in the leaf. In grasses they don't occur.

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was held temporarily by many persons (Ilkka Penttilä, Simo Salminen, Eero Haahti, Raimo Tenhunen, Reijo Vihko, Matti Härkönen, and Arto Pakarinen), because Tatu Miettinen, who was nominated to this position in 1972, never worked in the department, as he was also nominated professor of internal medicine at this university. At present the university staff of the Department of Clinical Chemistry consists of the professor, the associate professor (Matti Härkönen), one resident, two teaching laboratory technicians, one laboratory technician and one secretary. In addition, many scientists from other departments, two postdoctoral scientists from abroad, and several laboratory technicians paid with grants work in the laboratory. The department is responsible for the teaching of clinical chemistry to all medical and dentistry students at the university and also gives a separate complete course in clinical chemistry in Swedish. Furthermore, the department is responsible for the training of specialists in clinical chemistry and subspecialists in endocrinological laboratory techniques, haematology and nuclear medicine. Last but not least the department has formed an important research training environment for the more than 30 scientists who have carried out their dissertation thesis work (Ph.D.) during these 25 years.

To celebrate the 25th anniversary of the department, and the 350th anniversary of the University of Helsinki, the teachers of the department including the two professors and the 15 assistant professors (docents) of clinical chemistry, some of whom work in other clinics belonging to the Helsinki University Central Hospital or at Helsinki City hospitals, decided to publish this supplement containing overviews. These reviews reflect the main current interests of the authors, but also to a large extent the research areas of the department in the past. The diversity of topics and their actuality indicate that clinical chemistry at the University of Helsinki is in dynamic progress. Although molecular biology techniques are not yet reflected in these reviews the first papers have been submitted for publication.

This supplement was sponsored by the advertisers, to whom I wish to express our sincere thanks. The collaboration between the industry and the Department of Clinical Chemistry and the Central Laboratory, where also new instruments and reagents have been developed, has always been excellent. Finally, I would like particularly to thank the two editors of this supplement, assistant professors and divisional chief physicians Kristian Liewendahl and Ulf-Håkan Stenman, who have done an excellent job in editing the supplement, and Ulla Karjalainen, Ph.D., who has performed the page layout using QuarkXPress™ on a Macintosh II computer.

Helsinki (Helsingfors) March 1990

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Scand J Clin Lab Invest 1990; 50, Suppl 201: 3–23

Western diet and Western diseases: some hormonal and biochemical mechanisms and associations

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Adlercreutz H. Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest 1990; 50 Suppl 201: 3–23.

Breast cancer, prostate cancer, coronary heart disease and colon cancer belong to the so-called Western diseases and a general opinion is that diet is a significant or even the main factor increasing incidence and mortality of these diseases in the Western world. This review describes studies carried out in this department for about 10 years, many in collaboration with scientists abroad, and with the aim to clarify some of the connections between the diet and sex hormone, lipid and bile acid metabolism. A Western-type diet elevates plasma levels of sex hormones and decreases the sex hormone binding globulin concentration, increasing the bioavailability of these steroids. The same diet results in low formation of mammalian lignans and isoflavonic phytoestrogens. These diphenolic compounds seem to affect hormone metabolism and production and cancer cell growth by many different mechanisms making them candidates for a role as cancer protective substances. The precursors of these diphenols are to be found in fiber-rich unrefined grain products, various seeds, beans and probably also in pulses, peas and berries. Some types of fiber seem to influence sex hormone and bile acid metabolism mainly by partial interruption of the enterohepatic circulation, by alteration of intestinal metabolism and by increasing fecal excretion of these compounds. The sex hormone pattern found in connection with a Western-type diet is prevailing in the breast cancer patients, but is only partly a result of the diet.

Key words: breast cancer, prostate cancer, colon cancer, coronary heart disease, diet, fiber, lignans, isoflavones, estrogens, androgens, sex hormone binding globulin, dihydrotestosterone, bile acids, feces

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Breast cancer (BC), prostate cancer (PC) and endometrial cancer (EC) belong to the group of hormone-dependent cancers which in addition to colon cancer (CC), coronary heart disease (CHD) and some other diseases are called Western diseases because their incidence and mortality are high in the Western world compared to countries in Asia and South and East Europe [1–3]. In migrant studies an increased risk for Western diseases has been found to be related to a change towards a Westernized diet [4–9]. Migrants from Asia, Africa or East Europe to U.S.A. or Australia have

originally consumed a low-fat vegetarian or semi-vegetarian diet containing large amounts of unrefined carbohydrates. Most of these migrants and their children rapidly adopt a diet rich in calories, fat and proteins and low in complex carbohydrates and fiber [10] and their hormone [11, 12] and lipid levels change towards a Western pattern, increasing the risk for hormone-dependent cancer and CHD. Interestingly, migrants from a high risk colon cancer area (Scotland) to Australia experience a reduced risk for colon cancer [9].

Furthermore Hill et al. [13] postulated that a Western-type diet increases the concentration and metabolism of fecal bile acids (FBA) and neutral sterols (FNS), increasing the risk for CC. In the majority of the population studies carried out this hypothesis has rendered support with regard to the concentration but not with regard to the metabolism of FBA and FNS [review in 14]. On the other hand many animal experiments and *in vitro* tests have shown that free bile acids are cocarcinogenic or comutagenic [15, 16] but that the aminoconjugated bile acids may be inactive [16] in this regard [reviews in 14, 17]. It is believed that secondary bile acids are more toxic than primary ones and that a high litocholic acid (LCA) to deoxycholic acid (DCA) ratio is a CC risk factor [18–20].

Because of the obvious relationship between Western diet and Western diseases it has been postulated that this type of diet by some biochemical or other mechanisms may alter hormone production, metabolism or action at the cellular level increasing the risk for hormone-dependent cancer. Furthermore it has been suggested that the dietary composition may influence transit time of the intestinal content, fecal bulk and intestinal microflora and its environment causing alterations in concentration and metabolism of hormonal steroids, bile acids, neutral sterols, carcinogens and procarcinogens increasing the risk of CC and BC. Particularly in women, who have a much higher incidence of hormone-dependent cancer than men, diet has been suggested to be the main single determinant in the etiology of these cancers.

It is, however, very difficult to separate the effects of various single macro- or micronutritients on any biochemical event or steroid hormone or bile acid pattern or level. This is not only due to difficulties in the accurate recording of the diet, but also to the great variability in dietary intake during different seasons and even different parts of the week and the variability of hormone and steroid levels, particularly in women. Special efforts have to be made to standardize the conditions for sampling and to use reliable hormone assay methods and the recording of the diet must be carried out during sufficiently long time [12].

The following review will summarize and discuss results of our studies on the connection between diet and Western diseases. Many of these investigations are the result of collaborations with scientists abroad and some results discussed have not yet been published. The review will deal with some newly discovered mechanisms of dietary effects on sex hormone and intestinal bile acid metabolism and in addition with some interesting associations between the various diseases. Further support for the previously proposed extension [21] of the "fiber hypothesis" of Burkitt & Trowell [see 10] has now been obtained and will be discussed including not only BC and CC but also other Western diseases.

TABLE I. Abbreviations and trivial names of steroids and other abbreviations used in the text.

A	Androstenedione
BC	Breast cancer
CC	Colon cancer
CHD	Coronary heart disease
DHEAS	Dehydroepiandrosterone sulfate
Da	Daidzein
DCA	Deoxycholic acid
5α-DHT	5α-Dihydrotestosterone
EC	Endometrial cancer
End	Enterodiol
Enl	Enterolactone
E2	Estradiol
E3	Estriol
E1	Estrone
E1S	Estrone sulfate
Eq	Equol
FBA	Fecal bile acids
FNS	Fecal neutral sterols
For	Formononetin
FE2	Free estradiol
FT	Free testosterone
Gen	Genistein
2-OHE1	2-Hydroxyestrone
4-OHE1	4-Hydroxyestrone
%FT	Percentage free testosterone
%FE2	Percentage free estradiol
PC	Prostate cancer
LCA	Litocholic acid
LH	Luteinizing hormone
Mat	Matairesinol
SHBG	Sex hormone binding globulin
T	Testosterone

EFFECT OF VARIOUS MACRONUTRITIENTS ON SEX HORMONE METABOLISM

Effect of fiber

The development of a radioimmunological chromatographic method for the assay of the very low amounts of estrogens present in feces of men and nonpregnant women [22] made it possible for the first time to obtain a complete view of the effect of diet on the enterohepatic circulation of estrogens in man.

A high intake of fiber in premenopausal women increases fecal wet and dry weight, which correlates positively with all three unconjugated estrogens and total estrogens in feces [23]. In the same study also postmenopausal women were investigated (H. Adlercreutz, E. Hämäläinen, S.L. Gorbach, B.R. Goldin, J.T. Dwyer, M.N. Woods, unpublished results) and the same results were found. Furthermore, in the postmenopausal women we found positive associations between total fiber and grain fiber intake, and fecal estrone (E1) and estradiol (E2) excretion (list of abbreviations in Table I). Fat intake on the other hand seems to have a negative association with fecal excretion of estrogens [24] and therefore the dietary fat/fiber ratio of the postmenopausal women living in Boston shows highly significant negative correlation with fecal estrogen excretion (above-mentioned unpublished study). It is suggested that the dietary fat/fiber ratio determines the degree of interruption of the enterohepatic circulation of steroids, but the type of fiber plays also a significant role (see below).

In premenopausal women fecal weight and fecal estrogen excretion was found to correlate negatively with urinary estrogen excretion [23]. Particularly important was the observation of a negative correlation between fecal estriol (E3) and urinary E3-3-glucuronide (E3–3G) excretion. Urinary E3-3G is a specific metabolite of the intestinal mucosal cell and the end-product of estrogen metabolism and therefore a good indicator of the extent of the enterohepatic circulation of estrogens, particularly of E3 and other 16-hydroxylated and polar estrogens in man [25]. In a study carried out in Helsinki in premenopausal women it was found that total fiber intake and grain fiber intake/kg body weight were negatively associated with the excretion of 10 of the 13 estrogens measured in urine [26].

Fecal estrogen excretion shows a negative association with plasma E1 and E2 [23] and later on a direct negative correlation between total fiber intake and plasma E1 and E2 [24] and estrone sulfate (E1S) [27] could be observed in young women. Similar findings in men have been reported, but in addition to the negative correlation between crude fiber intake and plasma E2, higher fiber intake is associated with lower plasma testosterone (T) levels [28–30]. The reason for reduced intestinal reabsorption and increased elimination of estrogens by the fecal route in subjects consuming much fiber seems to be the larger fecal bulk and decreased concentration of intestinal β-glucuronidase [21, 23, 25]. The latter phenomenon reduces hydrolysis of the biliary steroid conjugates, an event necessary for their reabsorption. Some fibers have also the property of binding sex hormones, particularly non-polar estrogens [31, 32].

Preliminary results in the large study in Helsinki, called the "Finlandia study" revealed significant positive correlations between intake of total fiber, vegetable fiber and fiber from fruits and berries and plasma sex hormone binding globulin (SHBG) and negative associations between the intake of the same fibers and plasma % free estradiol (%FE2). Furthermore, total fiber, grain fiber and vegetable fiber intake correlated negatively with plasma % free testosterone (%FT) [33, 34]. The new results obtained in in postmenopausal Boston women [23, 24] agree well with the above-cited publications in that significant negative correlations were found between intake of total fiber, grain fiber and noograin fiber and plasma androstenedione (A), T, FT [35] and E1. In addition intake of fruit and vegetable fiber and grain calories correlated negative-

ly with plasma E1 [estrogen results unpublished, see 27].

It may be concluded that high fiber intake is associated with low levels of sex hormones in plasma, high SHBG and low %FE2 and %FT causing a reduction in the bioavailability of the hormones, which theoretically would reduce the risk of hormone-dependent cancer. The proposed mechanisms involved in changing the SHBG level will be discussed in the sections on dietary protein, and lignans and isoflavonic phytoestrogens.

Effect of protein

Most of the studies on the effect of protein intake on hormone metabolism have been carried out by altering the protein/carbohydrate ratio of the diet. Using this technique it was found that a high dietary protein/carbohydrate ratio decreases the plasma level of SHBG and T and that a low ratio has the opposite effect [36, 37]. Furthermore a high protein diet considerably diminished 4-ene-5α-reduction of T and enhanced 2-hydroxylation of E2 [38, 39]. By measuring the estrogen profile in urine by capillary GC-MS in premenopausal women [40, 41] we could recently confirm that a high dietary protein/carbohydrate ratio results in high urinary excretion of catecholestrogens. A new finding was that the dietary protein/carbohydrate ratio is highly significantly and positively associated with the urinary 2-OH-E1/4-OH-E1 ratio. Furthermore the lowest mean ratio (= 3.6) was found in vegetarians, followed by the omnivores (= 4.3) and the highest was found in the BC patients (= 7.1) (BC *vs.* vegetarians $p < 0.005$; BC *vs.* omnivores $p < 0.02$), who had the highest dietary protein/carbohydrate ratio due to low grain intake. It may be mentioned that this ratio was recently found to be 2.0 in the same Oriental migrant women in Hawaii [42], which were previously studied by us [24].

Effect of carbohydrates

In the above section the effect of changes in the dietary protein/carbohydrate ratio was discussed. Some further information as to the possible effect of carbohydrates on sex hormone metabolism derives from studies in which dietary intake of various macro- and micronutritients were correlated with plasma and urinary hormone levels.

Recently we found that postmenopausal women living in Boston showed significant negative associations between carbohydrate intake and plasma T, E1 and E2 [35,43]. Furthermore in the same study the intake of grain calories showed negative correlations with plasma A, T, DHEAS, and E1. The intake of carbohydrates also showed a weak but significant positive correlation with fecal E1 excretion (estrogen results unpublished).

In the corresponding Finnish study in 33 premenopausal women [40–42], studied twice during a year, we found some other interesting correlations between carbohydrates and sex hormones. Urinary 2-OH-E1/4-OH-E1 ratio correlated positively with protein/carbohydrate ratio of the diet and negatively with carbohydrate, starch, total fiber and grain fiber intake. Urinary 4-hydroxyestrone excretion correlated positively with total and grain fiber intake and plasma SHBG and negatively with %FE2 and %FT. Starch intake was negatively associated with urinary E3-3-glucuronide, the specific marker of the enterohepatic circulation of estrogens, suggesting partial interruption of this circulation in subjects with high starch intake. Carbohydrate intake was negatively associated with plasma E1S, the mean level of which was highest in the BC group. Plasma DHEAS on the other hand was strongly positively associated with plasma E1S, and less strongly with %FE2 and negatively associated with urinary 16-hydroxylated estrogens and enterolactone (Enl) [27]. Enl mainly derives from precursors in grain and its urinary excretion reflects both the intake of fiber in general [44] and whole-grain products in particular. The results indicate that it is difficult to separate the effect on hormone metabolism of complex carbohydrates from that of fiber.

Effect of fat

Oriental women living in East Asia and at low risk for BC consume a very low-fat diet (usually < 20 % of calories). Studies on the urinary excretion of E1, E2 and E3 have shown that they excrete lower amounts of E1 and E2 and similar amounts of E3 compared to women in Western countries [24, 45, 46]. In other studies in vegetarians living in Western societies the picture has not been so clear, but there has been a trend towards lower urinary E1 and E2 values and similar or slightly higher E3 values in the vegetarians [47, 48]. Thus a vegetarian or semivegetarian diet seems to be associated with relatively high E3 formation. The simultaneously higher fecal excretion of E3, however, reduces urinary E3 levels leading to varying quantitative results for E3 in urine, depending mainly on the nature of the fiber in the food and the quantity of both dietary fiber and fat. Simultaneously there seems to be a reduction in the relative concentration of 2-hydroxyestrogens, particularly in Oriental women and a relative increase in 4-hydroxylation [41, 42], which means that the main metabolic pathways in these women unexpectedly seems to lead to biologically more active estrogens. However, it must be remembered that their plasma and urinary E1 and E2 levels were shown to be low [24] and the net biological estrogen effect may in any case be less. It has also been shown that the luteal phase E2 values are lower in young women following a low-fat diet for 2 months [49].

Women living in Africa consuming low-fat habitual diets [50] and Oriental migrants in Hawaii [24] have low plasma androgen levels compared with women on a Western diet. These observations are in agreement with the results obtained in postmenopausal omnivorous and vegetarian women and postmenopausal women with BC showing the lowest plasma A, T, %FT, %FE2 and DHEAS and highest SHBG (after correction for weight) in the vegetarian women, who had the lowest dietary fat/fiber ratio of the three groups [35, 43] The lower DHEAS in vegetarians is in agreement with recent results showing that plasma E1S levels are lower in women on a low-fat high-fiber diet compared to a typical Western diet [51] because the levels of these sulfates show a significant association [27 and unpublished results]. In correlation analysis a Western-type diet was found to be associated with the hormonal pattern observed in the postmenopausal women with BC, but this was obviously not entirely due to the diet [35].

It seems justifiable to conclude that a high protein and fat and low grain, complex carbohydrates and fiber intake leads to higher plasma levels of biologically active sex hormones and lower SHBG, with a clear tendency to lower 16α– and 16β-hydroxylation [42] and higher 2-hydroxylation of estrogens and higher urinary 2-hydroxy-E1/4-hydroxy-E1 ratio. The possible role of these alterations of hormone levels as etiological factors in hormone-dependent cancer will be discussed below. It should be mentioned that opposite results with regard to 16α-hydroxylation of estrogens and fat intake have been published [52, 53], and these results will be discussed in the section on BC.

LIGNANS, ISOFLAVONES, AND SEX HORMONE METABOLISM

Since the detection and identification of mammalian and later also of plant lignans and isoflavonic phytoestrogens in the human organism, many studies on their biological role in health and disease have been carried out. Several reviews [33, 54–56] on the topic have recently been published. These diphenolic compounds occurring in high amounts in the organism have numerous different biological activities of which most seem to make them candidates for a role as protective substances with regard to cancer and particularly hormone-dependent cancers [12, 21, 33, 34, 54, 56–64].

To date 15 lignans and isoflavonic phytoestrogens, all diphenolic in character, have been identified in human urine and some of them also in other biological materials [54, 56, 65, 66]. Of these 7 can now be measured by combined capillary gas chromatography–mass spectrometry utilizing the selective ion monitoring technique and isotope dilution mass spectrometry using deuterated internal standards [58, 67]. The lignans enterolactone (Enl), enterodiol (End) and matairesinol (Mat) and the isoflavonic phytoestrogens daidzein (Da), equol (Eq), O-desmethylangolensin (O-Dma) and genistein (Gen) have all weak estrogenic activity, but antiestrogenic activities have also been described [reviews in 54, 56]. Many plant lignans have been shown to have anticarcinogenic, antiviral, bactericidic and antifungal activities. In collaboration with Dr Larry Vickery (Irvine, California) it was shown that Enl and a theoretical

intermediate between Mat and Enl are moderate inhibitors of placental aromatase and compete with the natural substrate androstenedione for the enzyme. Enterolactone was also able to inhibit aromatase intracellulary in cell cultures suggesting that these compounds may function as natural aromatase inhibitors. Other experiments show that these diphenols are readily transferred from cell culture media into the cells and that they may inhibit cancer cell growth, because antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast cancer cell line, ZR-75-1, were reported [59]. Furthermore, inhibitory effects of such compounds on mitogen-induced proliferation of human peripheral blood lymphocytes were demonstrated [60].

Genistein, one isoflavonic compound identified by us in human urine is a specific inhibitor of tyrosine-specific protein kinases [61-64]. Protein-tyrosine kinase activity is associated with cellular receptors for epidermal growth factor (EGF), insulin, insulin-like growth factor I (IGF-I), platelet-derived growth factor (PDGF) and mononuclear phagocyte growth factor (CSF-1), suggesting that the enzyme plays a role for cell proliferation and transformation. The enzyme has also been associated with oncogene products of the retroviral src gene family and is correlated with the ability of retrovirus to transform cells [literature in 61–64].

In collaborative studies with Dr Jim Clark and associates we have found that several plant and mammalian lignans and isoflavones compete with E2 for the rat uterine nuclear estrogen type II binding site (unpublished results). These sites seem to constitute a component of the genome which regulates estrogen-stimulated uterine growth [68, 69]. It was found that some flavonoids like luteolin, quercetin and pelargonin inhibit E2 binding to this receptor and in this way uterine cell growth. They also inhibited growth of MCF-7 cells in culture, and *in vivo* E2 stimulation of immature rat uterus [70]. The structure of these compounds are very similar to those of the isoflavones and in fact all are diphenols. The most effective with regard to type II site binding of the diphenolic compounds found and measured by us in human urine seem to be the isoflavones daidzein and equol, but also some lignans like matairesinol, isolariciresinol and enterolactone show competition (competition observed at concentrations from 10 to 100 nmol/l). Later an endogenous inhibitor of the nuclear type II binding site was identified as being methyl p-hydroxyphenyllactate [71], which can be a metabolite of both exogenous flavonoids and tyrosine. Because this compound cannot be found in cancer tissue it was postulated that uncontrolled growth and proliferation of malignant cells is directly related not only to the permanent stimulation of nuclear type II binding sites by estrogens or other compounds, but also to very low to nonmeasurable levels of the competitive inhibitor methyl p-hydroxyphenyllactate [71]. In our opinion it seems that probably many of these phenolic compounds may have a synergistic action as it is unlikely, because of close structural similarities, that only one of them inhibits cell growth. The compound found by Markarevich et al. [71] was isolated from fetal bovine serum, probably a very rich source of many flavonoids and phytoestrogens and their metabolites. The concentration of the new monophenolic compound in biological fluids and tissues in human subjects has to my knowledge not been measured. The possible growth-inhibiting and antiproliferative role of individual flavonoids and their metabolites with regard to hormone dependent cancer is a new interesting area of research that needs much further studies.

Of the isoflavones the strongest estrogens are Eq and Gen, but they are still very weak estrogens compared to E2 and E1. It is unlikely that all their other biological effects are related to their estrogenicity. Quantitative results indicate that lignans and isoflavonic phytoestrogens are normal constituents of human urine and are excreted in large amounts particularly by vegetarians (both lignans and phytoestrogens) [33, 34, 58], by subjects consuming large amounts of whole-grain products, vegetables and berries, which all are associated with high lignan excretion [33], and by the Japanese consuming traditional Japanese diet (mainly isoflavonic phytoestrogens, due to intake of soy products) [33, 72]. In omnivorous Finnish subjects the excretion of Gen, the specific inhibitor of protein tyrosine kinase, was found to be between

10 and 1,500 nmol/24 h (usually 1-4 times that of Da). When investigating a few Japanese subjects consuming a traditional diet the excretion was very high ranging from 1,250 to 15,500 nmol/24 h (!) (in collaboration with H. Honjo and coworkers), about 1.5 - 3 times higher than that of Da. As mentioned Da shows antiproliferative activity with regard to BC cells [59]. Particularly low excretion of these compounds has been observed in BC patients and in subjects consuming a low-fiber diet, especially a diet low in whole-grain products and beans [23, 24, 49, 64, and unpublished results]. Particularly low excretion has been observed in BC patients and in subjects consuming a low-fiber diet, particularly a diet low in whole-grain products [33, 34, 58, 73].

It has now been demonstrated that the mammalian lignans Enl and End are formed from precursors, such as the plant lignans matairesinol and secoisolariciresinol, which are consumed and then structurally modified by intestinal bacteria [56]. Eq and O-Dma are most likely formed by intestinal bacterial action from formononetin (For) and Da present in food stuffs like soy products [72, 74]. However, these compounds are also present in cow milk [75] formed from *e.g.* For in clover by intestinal bacteria in the gastrointestinal tract of the cow [55], and may therefore be consumed by human subjects as such. Because of the close association of lignan excretion with fiber intake [21, 33, 44] it is likely that the plant lignans are localized close to the outer fiber-containing layers of the grain containing phytin, polyphenols, enzyme inhibitors and other compounds usually regarded as antinutritional factors [76].

Recently, we suggested that the lignans and isoflavonic phytoestrogens, which all are diphenols, perhaps together with other similar compounds, stimulate SHBG synthesis in the liver and in this way reduce the biological effects of sex hormones [27, 33, 34]. An increase in SHBG results in lowering of %FT and %FE2 and reduction of both the albumin-bound and the free fraction of the sex hormones. This reduces the metabolic clearance rate (MCR) of the steroids and reduces in this way their biological activity.

In Finnish women total fiber intake, total fiber intake/kg body weight and grain fiber intake/kg body weight correlate positively and dietary fat/fiber ratio negatively with urinary excretion of total lignans and isoflavonic phytoestrogens [33, 34]. The excretion of the two diphenolic groups of compounds and also Enl alone in both pre- and postmenopausal Finnish women correlate positively with plasma SHBG and negatively with plasma %FE2 and %FT [33, 34 and unpublished results]. It is well known that oral estrogens, in contrast to parenterally administered ones, markedly stimulate SHBG synthesis [77, 78] and we therefore suggest that these positive associations between urinary lignan and phytoestrogen excretion and SHBG is due to stimulation of SHBG synthesis by these week estrogens entering the portal circulation in very high amounts. This also would explain the higher SHBG values seen in vegetarians [79] including such vegetarians whose diet does not contain low amounts of proteins [34]. High protein diet has been found to lower plasma SHBG [36, 37].

Furthermore urinary Enl excretion in these Finnish women correlates negatively with plasma DHEAS and luteinizing hormone (LH) (unpublished observations). The latter observation has to be evaluated in detail, but it is possible that the effect on sex hormone metabolism of these weakly estrogenic compounds may also be mediated via an effect on the hypothalamic–hypophyseal endocrine system. Plasma DHEAS is low in vegetarians and is negatively associated with the dietary intake of unsaturated fatty acids [35].

DIET, SEX HORMONES AND BREAST CANCER

In an extensive review about 10 years ago Dao concluded that studies of estrogen metabolism in BC has provided only controversial results and that they are inconclusive at best [80]. The results described above indicate clearly that studies on sex hormone metabolism in cancer cannot be carried out without careful dietary evaluation in the subjects studied. It is therefore not surprising that no consensus as to the association between sex hormone changes and BC has been reached, because very few studies include both detailed dietary records and hormonal investigations.

In his recent review Zumoff [81] includes nine hormone-related hypotheses in the discussion on hormones and BC, but none of them was discussed in relation to diet despite the huge amount of epidemiological data suggesting that a Western diet plays an essential role in increasing the BC risk in the Western world.

Because of the extensive literature I will discuss only a few of those hypotheses regarding the association of sex hormone alterations and BC, which seem to be related to diet.

The main change in diet when subjects from developing countries migrate to Western countries is an increase in animal fat and protein and a decrease in intake of complex carbohydrates, particularly whole grain products [10]. This change is identical to what has occurred in Scandinavia in the last 300 years and in fact has been going on in Finland since World War II with a simultaneous increase in the incidence of BC, CC and other Western diseases. I therefore like to discuss particularly the possible role in cancer development of complex carbohydrates like whole grain products and soy beans, cereal fiber and the role of lignans and isoflavonic phytoestrogense and their association with plasma SHBG and the % free sex hormones.

In two case-control [82, 83] and in an epidemiological study [84] it was shown that high fiber and high carbohydrate intake, respectively, decreased the risk of BC. In another case-control study particularly fiber from grains consumed during adolescence reduced the risk both in premenopausal and postmenopausal women [85]. These observations are in agreement with the results of our studies in postmenopausal women in Boston [35] and in premenopausal women in Helsinki [41] showing that the main and in fact only really significant difference between the diet of the BC patients and the omnivorous and vegetarian control women was a low intake of grain products and grain fiber. If the diets of the Boston and Finnish women studied by us are compared, the main difference is also in the grain and grain fiber intake, being much higher in the Helsinki women with a lower risk for BC than the Boston women. This dietary difference caused the mean fecal weights to be higher in the Finnish compared to the Boston women, despite similar mean total fiber intakes. The large fecal bulk affects the enterohepatic circulation of sex hormones, because there is *e.g.* a significant correlation between fecal weight and fecal estrogens. In both countries the fat/fiber ratio was the same in the omnivorous and BC women, but much lower in the vegetarian women, particularly in Boston, because the Finnish vegetarians consumed rather much fatty milk products. The postmenopausal Boston BC women had lower fat intake than the Finnish young vegetarians (!), the protein intake being similar. However, the fat to grain fiber ratio (g/g) was 16.4 in the old Boston BC women and only 10.2 in the young Finnish BC women and the corresponding values for the omnivores were 15.1 and 8.2, respectively. The Boston and Helsinki vegetarians had total fat/grain fiber ratios of 7.1 and 6.3, respectively. Very interesting are also the results of the protein/grain fiber (g/g) ratios in the six groups of women. The vegetarians, omnivóres and BC patients in Boston and Helsinki had the following ratios: 7.2, 15.2, 18.1, and 5.4, 7.2 and 8.8, respectively. This shows that these ratios are very high in the omnivorous women and the BC patients in Boston, and also highest in the BC group in Helsinki compared with the other Finnish women mainly due to differences in grain fiber intake.

The fat intake in the BC women both in Boston and Helsinki was intermediate between that of the omnivores and vegetarians in respective city. This may be due to bias, particularly in Helsinki, because of much propaganda in this country about reducing fat intake in order to avoid cancer and other diseases. However, small differences in fat intake will not have any detectable effect on plasma or urinary sex hormone levels (for discussion see [12]), which may to some extent explain the results of a recent prospective study [86] in nurses that failed to show any correlation between high fat consumption and the subsequent development of BC (see also [87, 88]. However, in our opinion, after considering our above-mentioned results, it seems more appropriate to use the fat/total fiber or fat/grain fiber ratio to define the diet of risk groups and controls than to use % fat calories or total fat intake. However, recent prospective studies in our laboratory suggest that

particularly grain products containing all compounds of the grain may be protective and that so-called whole-meal products may be less satisfactory in this sense (see below). Also protein/carbohydrate or particularly protein/total fiber or protein/grain fiber ratio should perhaps be used to define the dietary groups. Using such ratios we have observed that the association of diet to sex hormone metabolism becomes much more obvious. We believe that this is related to the intestinal metabolism of hormones, lignans and isoflavones, which is dependent on the intestinal environment and closely related to our diet and perhaps better described or reflected by these ratios than by expressing the amounts of macronutritients as percentages of total calories or in relation to body weight.

Without doubt it is not fat alone which has the negative effects on overall sex hormone levels, but proteins, fiber and complex carbohydrates seem at least in Western societies to play even more essential roles. As an example of what this concept means is the increasing effect of a high fat and meat and low grain intake both in man and in experimental animals on intestinal β-glucuronidase (see literature in [21, 23]), which theoretically leads to an increase of the reabsorption of estrogens from the intestinal tract [25] and higher plasma estrogen levels [23, 24]. It should also be emphasized that the associations between fiber intake and the excretion of a number of urinary estrogens became statistically significant first when the fiber/kg body weight ratio was used instead of total fiber intake [26]. The fiber/kg ratio may better reflect the intestinal bacterial environment and fiber effects because a small subject has a smaller "internal" volume of the intestines compared to a tall subject.

The diet in Finnish rural areas where BC and CC incidence is low differs from the American one particularly with regard to its relatively high content of complex carbohydrates mainly from whole-grain products and starchy vegetables, the fat content being similar but deriving more from milk products than from meat [89, 90 and own observations]. A significant part of the Finnish milk product consumption consists of fermented milk products. Because of the differences in BC risk in USA and Finland we have postulated that this difference is at least partly due to the great difference in intake of whole-grain fiber-rich products like rye bread and perhaps some other fiber-rich nutritients such as berries. Particularly these foodstuffs increase the excretion of urinary lignans by the Finns and affect simultaneously also otherwise the intestinal milieau. This view was supported by the finding of very low urinary lignan excretion in the BC subjects living in Boston [57] and of lower excretion also in the young BC women in Helsinki [34, 73]. In both BC groups it was likely that the differences were due to low intake of whole-grain products. However, in Helsinki the differences between the omnivorious, vegetarian and BC groups were relatively small, because the grain intake was comparably high in all groups, which is typical for the original Finnish diet. It should be mentioned that the intake of wheat germ and bran do not at all cause increases in urinary lignan excretion in human subjects (own observations), and fiber-free wheat bread products have no or only very small influence on lignan excretion. Only grain products which have been made from milling of whole grain, without separating (and washing) the different components and mixing them again (R. Korpela and H. Adlercreutz, to be published) seem to significantly increase lignan excretion in Finnish women. This is because during modern milling of the grain, trying to eliminate so-called antinutritional factors [76], simultaneously also the diphenolic plant lignans seem to be at least partly eliminated. There are indications that also berries, fruits and various seeds [33, 56, 91] increase lignan excretion. Of some grain products, rye meal seems to result in the highest excretion of lignans in rats, followed in decreasing order by oat, barley and wheat meal [91]. The latter results are difficult to evaluate because no exact details were presented regarding the nature of the meal products consumed by the rats.

Based on an epidemiological study it was recently suggested that consumption of fermented milk products may protect against breast cancer [92]. In a case-control study consumption of fat from milk, cheese and yogurt during adolescence reduced the BC risk both in premenopausal and postmenopausal women [85]. One mechanism by which fermented milk may influence hormone metabolism

is by reduction of the β-glucuronidase-producing bacteria of the intestinal content [93, 94], which theoretically should reduce the enterohepatic circulation of estrogens and increase the fecal route of elimination. The conjugated estrogens excreted in the bile must be deconjugated before the estrogen moiety can be reabsorbed. Milk products have also been found to contain animal lignans and isoflavonic phytoestrogens (75) and even if the concentrations are rather low they add to those produced by the intestinal bacteria from plant precursors.

Our hypothesis has been that high intake of whole-grain products (preferably in combination with reduced fat and moderate protein intake) reduces BC (and CC) risk because such a diet increases fecal bulk and reduces intestinal β-glucuronidase activity and steroid and bile acid enterohepatic circulation and results in increased mammalian lignan production [12, 21]. Later on we also included the isoflavonic phytoestrogens into the original theory [33, 54]. This was due to the finding of very high excretion of isoflavonic phytoestrogens in urine of Japanese men and women consuming a traditional diet [33, 72]. The lignan excretion in the Japanese subjects was low, even lower than we found in the postmenopausal BC patients in Boston. The isoflavones resemble lignans with regard to structure (all are diphenolic). In most correlation studies they show parallel behaviour. In the Finnish women the significances of the positive correlation between the excretion of lignans and isoflavonic phytoestrogens in urine, and plasma SHBG, and the negative correlations with %FE2 and %FT are stronger than the separate correlations for each group of compounds [33]. Recently, our hypothesis with regard to the protective role of these compounds for BC got strong support from studies showing that powdered soy bean chips, both before and after denaturation of protease inhibitors, decrease mammary tumor formation in a rat breast cancer model [95]. Furthermore Gen, found by us in human, chimpanzee and cow urine, may be anticarcinogenic due to its inhibitory effect on protein tyrosine kinase [61–64] and other flavonoids are antiproliferative with regard to BC cells [59]. The postmenopausal BC patients in Boston had the lowest plasma SHBG and highest %FT and %FE2 [35] and the lowest Enl and Eq excretion [57]. The Finnish premenopausal BC subjects had lower SHBG, higher %FT and %FE2 and lower excretion of lignans and isoflavonic phytoestrogens compared to the vegetarians [34]. In many studies low SHBG has been associated with BC (see literature in [35, 96]).

Because of the large differences in grain fiber intake and urinary lignan excretion between postmenopausal women living in Helsinki and Boston we have in preliminary calculations combined the materials of postmenopausal women and found the same highly significant positive correlation between grain fiber intake or Enl excretion and plasma SHBG and negative correlations with plasma %FE2 and FT (unpublished observations) as we found for the young Finnish women [33, 34].

The theory based on the observation that high fat intake increases 16α- and decreases 2-hydroxylation of estrogens leading to biologically more active estrogens also needs some discussion. According to this theory a low rate of 2-hydroxylation and high rate of 16α-hydroxylation leads to a greater risk for BC and endometrial cancer [52, 53, 97–99] because 2-hydroxylated estrogens are biologically less active than 16α-hydroxylated ones. Several earlier studies as well as our own seem to speak against this hypothesis because all low-risk groups, compared to high-risk groups, have relatively more urinary 16α-hydroxylated estrogens, particularly if also the fecal estrogens are included. Women living in low-risk countries consume most of their calories in the form of complex carbohydrates and have lower fat and protein intake, which should lead to low 2-hydroxylation of estrogens [38, 39]. This we could observe in the young premenopausal Finnish women [40, 41] and in the previously investigated Oriental women [23, 42]. The characteristics of the sex hormone pattern in these low-risk Oriental women on a low-fat diet are low plasma levels of E1, E2, A and T and low excretion of E1, E2 and 2-hydroxylated estrogens and relatively high amounts of both 16α- and 16β-hydroxylated estrogens [23, 42] We could also not see any increase in 16α-hydroxylated estrogen metabolites in urine of Finnish premenopuasal women with BC. In fact slightly higher mean values were seen in the vegetarians, but the differences were not significant [40, 41].

Recently we completed the second part of the Finlandia study dealing with groups of postmenopausal women and found results apparently more in line with those suggesting that high 16α-hydroxylation is a risk factor. A statistically significant (logarithmic) negative correlation between plasma SHBG and urinary 16α-hydroxyestrone ($R = 0.59$, $p < 0.001$) and estriol ($R = 0.49$; $p < 0.01$) was found with the highest values of estriol and lowest SHBG values in the BC and omnivorous women and higher SHBG and lower urinary 16α-hydroxylated estrogens in the vegetarians. In the same material there was a significant positive correlation between urinary total diphenol excretion and plasma SHBG ($R = 0.64$; $p < 0.001$). From our results it appears that the tendency to lower values of 16α-hydroxylated estrogens in urine of the vegetarian and higher in the omnivoric and BC women is probably due to different degrees of fecal elimination of these estrogens as a result of differences in fiber intake and not to increased 16α-hydroxylation of estrogens in BC. However, the evaluation of this very large study is still in progress and the definite results have to await the extensive statistical treatment needed. In these postmenopausal women we found no correlation between plasma SHBG and urinary catecholestrogens but a highly significant positive association between the logarithms of plasma E1S and urinary excretion of 2-hydroxy-E1 ($R = 0.84$; $p < 0.001$). The BC women tended to have both higher plasma E1S and urinary 2-hydroxy-E1, which supports our theory that high E1 and E1S and urinary catecholestrogens may be risk factors of BC. It may be mentioned that high E1S has also been found in EC [100].

With regard to 2-hydroxylated estrogens there is evidence speaking for a role of these steroids and catecholestrogens formed from stilbestrol in hormonal carcinogenesis via microsome-mediated redox cycling and formation of quinones and free radicals [101]. The quinoid structures are prerequisites for the genotoxic effect [102] because they are capable of covalent binding to proteins [103, 104]. The development of renal tumors in Syrian hamsters after estrogen treatment has been postulated to occur via a free radical mechanism [105]. Hydroxylated flavonoids have antagonistic effects on the mutagenic and/or tumorigenic activity of epoxide metabolites of polycyclic aromatic hydrocarbons [106]. Because of similar structure the isoflavones and lignans should also be investigated in this respect.

DIET, HORMONES, LIGNANS AND ISOFLAVONES, AND OTHER WESTERN DISEASES

It is not possible in this connection to discuss at any length the relationships between diet and other Western diseases. Some very large reviews on nutrition and its relationship to cancer have been published [107, 108]. However, I would like to discuss shortly some new results indicating that the above discussion may have some important implications also for other diseases than BC and that obvious hormonal and biochemical connections exist between BC and other Western diseases.

Endometrial cancer

What has been said about diet and estrogen metabolism and BC holds as well for EC, a disease even more clearly estrogen-dependent than breast cancer. An increase in bioavailable estradiol due to lowering of SHBG and increase in reabsorption of biliary estrogens as a result of a Western diet would also promote the growth of endometrial cancer. This cancer type has in addition been found to be associated with other diseases common in the Western world, like hypertension and diabetes. Hypertension has in fact recently been found to be a risk factor also of BC [109].

Prostate cancer

Furthermore, it is known that a low-fat and/or high-fiber diet affects sex hormone metabolism also in men [28–30] by decreasing T and FT. A high level of biologically active androgens probably accelerates the development of PC in the Western world and recently a prospective study in fact seems to indicate that elevated T levels are

associated with increased risk of PC [110]. In epidemiological studies fat and meat show a positive and cereals a negative association with PC mortality [3]. In Japan and some other Asian countries, despite the same incidence of latent small or non-infiltrative prostatic carcinomas, the mortality is low [111–113]. This could at least partly be explained by a diet-related lowering of biologically active androgens as seems to occur in Asian women [24] and in the above-mentioned experimental studies [28–30]. Rotkin (cited from [114]) suggested that the men at risk of developing PC had a "strong overbalance of androgenic components" and observed that fewer patients with prostatic cancer developed gynecomastia and obesity early in life compared to controls. However, also recent observations indicate a possible protective effect of endogenous estrogens [115, 116] and this would suggest that the high levels of isoflavonic phytoestrogens in the traditional diet of Japanese men [33, 68] may also represent a protective factor [33, 54] inhibiting the growth of already existing small cancers [theory originally proposed in 117]. However, other than estrogenic effects of these substances may be more important.

The above-mentioned theory gains support from the recent observations of decreased risk of prostate cancer in Adventist men showing high consumption of beans, lentils, peas and some dried fruits (dietary sources of flavonoids) [118] and in men of Japanese ancestry in Hawaii consuming much rice (mainly starch, which may have some fiber-like effects in the gut) and tofu [119], a soy bean product. Our own results in Japanese men and women [some results in 72] show a strong positive association between the intake of various soy products and urinary excretion of equol and daidzein, and also a positive correlation with lignan excretion, particularly enterodiol, despite the fact that lignan excretion was low in the Japanese subjects investigated. It was in fact suggested [112], that if new small latent carcinomas are being formed at a constant rate they may either disappear or may enlarge and develop into larger carcinomas in different numbers or at different speeds in different geographical areas. It is suggested that in certain populations dietary factors affect androgen metabolism and biological activity as described above and/or that dietary isoflavones and other phytoestrogens directly influence cancer cell growth slowing the speed of development of these small latent carcinomas. The possible effect of soybean diets on PC may be a parallell to the observation of the inhibitory effect of this diet on breast tumor incidence in experimental animals [95, 120].

Coronary heart disease

Low SHBG has been found to be a risk factor of CHD mortality in a female population during a 12-year follow-up period [121] and is probably a risk factor also in men [122]. In addition, low plasma 5α-DHT seems to be a risk factor of CHD in men [122, 123]. As mentioned previously a high dietary protein/carbohydrate ratio not only suppresses plasma levels of SHBG, but simultaneously inhibits liver 5α-reductase [36–39]. Furthermore, we found significantly higher SHBG and HDL-cholesterol and almost significantly higher 5α-DHT ($p < 0.07$) in joggers compared to the subjects with CHD and a positive association between SHBG and HDL-cholesterol and maximal oxygen uptake in both joggers and healthy men [122]. Plasma SHBG and 5α-dihydrotestosterone concentration correlates positively with HDL-cholesterol and apoliprotein A-I both in healthy middle-aged men and in men with CHD [122, 123]. It is also known that thyroid hormones and estrogens stimulate SHBG synthesis, increases liver 5α-reductase and plasma HDL-cholesterol and apolipoprotein A-I [124, 125]. In population studies HDL-cholesterol and apolipoprotein A-I are inversely related to CHD [126, 127]. Compounds increasing the 5α-/5β-reductase activity ratio in rat liver microsomes lower serum cholesterol and reduces the incidence and severity of atherosclerotic lesions in aortas of cholesterol-fed rabbits [128]. Whether the higher plasma SHBG and 5α-DHT in our physically fit men compared to the subjects with CHD is due to diet or to physical exercise itself cannot be judged at present. The protein/carbohydrate ratio of the diet may be lower in hard-training joggers, which could explain the high SHBG and 5α-DHT levels. This is because aerobic training usually leads to increased proportion of carbohydrates in the diet.

In Finnish men this may mean increased consumption of whole-grain rye products, because about 40 % of the cereals consumed in Finland are rye products [see *e.g.* 129] and rye bread is usually a whole-grain product in this country.

As already mentioned consumption of whole-grain rye bread has recently been found by us to considerably increase animal lignan excretion in urine (R. Korpela, H. Adlercreutz, to be published), and it also seems to stimulate SHBG synthesis (almost statistically significant increase after 2 weeks; $p < 0.07$) as suggested previously [33]. Furthermore, it is of interest that isoflavones, excreted in high amounts in urine in populations having a low CHD risk, like the Japanese men, have hypocholesterolemic effects in rats [130] and that treatment with a soybean-protein diet has remarkable hypocholesterolemic effects in human subjects with type-II hyperliproteinemia [131]. Soybean protein products contain isoflavonic phytoestrogens, but whether the effect observed is due to these compounds or to the plant protein itself, as suggested by the authors, is uncertain. It is interesting to note that it has been suggested that the hypocholesterolemic effects of isoflavones is probably independent of the estrogenic effects [130]. Furthermore, it has been shown that the hypocholesterolemic effect of soy products in human subjects is not due to the content of soybean fiber [132]. It is concluded that very similar associations between diet, SHBG, lignans and isoflavones, as found for BC, seem to exist also with regard to CHD.

Colon cancer

In epidemiological studies a parallelism has been observed between BC and CC [133], but there are also some discrepancies suggesting different etiology [review 86]. However, for none of the Western diseases the etiology is likely to be monofactorial and looking only for the associations with macronutritients may easily lead us to wrong conclusions. There are also some parallelisms between CC and PC [3], and diet, in the majority of the opinions [107,108], seems to be the most important environmental factor in the development also of CC.

CC has also been found to be related to reproductive and hormonal factors [review in 130] and it has been found that increasing parity decreases risk and late age at first live birth increases risk [135, 136] as found also for BC. Women with cancers of the breast and other reproductive sites have an excess of primary colorectal cancer and pregnancy protects against DMH-induced colon cancer in experimental animals [review in 136]. Many colon tumors contain sex hormone receptors [137-140], and they may play a role in the pathogenesis of the disease [141].

The observed discrepancies in parallelism between CC and BC incidence and mortality development in Japan [86] may be due in addition to changed consumption of macronutritients to some micronutritients like plant lignans and isoflavones having a large spectrum of biological activities like anticarcinogenic, antiproliferative, antihormonal or hormonal and antiviral effects, which may play a role also locally in the intestine [21, 142]. The local effects in the intestine may be independent of the formation of the hormonally active substances which seem to alter liver and peripheral sex hormone metabolism. Another factor which may play a role for the discrepancies in parallelism between CC and BC is that a change in the fat content of the diet e.g. in Japan may not parallel a change in the use of soy products, because the soy sauce is mainly used for its content of sodium chloride and other soy products may still be used independently of an increase in fat intake. When leaving the habit to consume a low-fat diet the Japanese seem to still consume rice and they do not get any additional (cereal) fiber needed to compensate for the higher fat intake, because whole-grain bread seems to be almost unknown in Japan. This in our opinion could perhaps explain that the CC incidence in Japan increases more rapidly than the BC incidence [86] because of the absence of cereal fiber but continuous consumption of soybean products and rice.

Furthermore, an increase from 10 to 25 % of the fat calories as has occurred in Japan between 1955 and 1975 [86] may not alter the hormonal pattern as much as the difference we find for urinary and plasma sex hormones when the fat calory intake is

about 20 % compared with that found when it is about 38 % [24, 42]. In our own studies in a rural village outside Kyoto [72] women and men still consume only 20 and 17 % fat calories, respectively.

In most epidemiological studies a relation between fat intake and CC has been observed, but in only few studies an association has been found between CC risk and high protein intake or high energy consumption [143, 144] both leading to low SHBG, despite the fact that fat and protein consumption generally increase in parallel. In one study a high meat/vegetable consumption ratio predisposed for CC [145], a diet, which probably also would affect sex hormone pattern [35].

However, as for BC, a negative association between CC and intake of cereals or nonstarch polysaccharide fiber has been observed in most (but not all) epidemiological studies (review in 146, 147], the case-control studies being less convincing [see 147]. To my knowledge no prospective studies on effect of grain fiber or whole-grain products on CC incidence have been published. Recent studies suggest that the fat/fiber ratio is important also in the pathogenesis of CC because a negative association between CC and dietary fiber was found only in men with low fat consumption [148]. Epidemiological studies in Finland and Denmark point to a protective role of cereal fiber [89, 90, 129, 149], but also other factors like high consumption of fermented milk lowering colonic pH [150, 151] and supplying calcium [152, 153] [review in 154] are most likely partly responsible for the favourable CC incidence in rural Finland. Thus fermented milk may play a role for both BC (lowering effect on intestinal ß-glucuronidase) and CC risk [94, 154, 155]. As indicated above the dietary fat/fiber ratio seems to determine the degree of the enterohepatic circulation of hormonal steroids and may in this way alter the risk of hormone-dependent cancers. In experimental colon carcinogenesis this ratio determines the tumor prevalence and dietary fiber content determines the bile acid concentration and protects against the deleterious effects of fat [156, 157].

Because of the relatively high consumption of whole-grain rye bread in Finland we have been interested in studying whether different cereal products may have different effects on the CC risk factors. From these studies we have now obtained more support for the theory [21] that certain fiber-rich grain products, supplying precursors for mammalian lignan formation perhaps protecting against BC and locally having a favourable influence on intestinal bacterial composition and metabolism and mucosal cell environment, may be protective with respect to CC also by another mechanism. This is because rye bread seem to favourably influence intestinal bile acid metabolism. In a recent experiments we observed that by changing the bread consumption from a wheat fiber-free bread or from a whole-meal fiber-rich (fiber > 9 %) wheat bread to a whole grain rye bread (fiber > 8 %), significant alterations of the biochemical risk factors of CC could be obtained (see below), suggesting that the relatively small dietary change may have positively affected intestinal metabolism. The rye bread made from whole grains, not purified during milling, compared to both the fiber-free and a fiber-rich wheat bread (produced after modern milling of the grain eliminating some fractions, but containing essentially all components) increased considerably the urinary lignan excretion (R. Korpela & H. Adlercreutz, to be published). Compared to the control period no change (whole-meal) or a decrease (wheat, fiber free) was observed for the other breads. As mentioned above we have shown that lignan excretion is low in women with BC [21, 34, 57], most likely due to low intake of whole-grain bread. Furthermore, it has been shown that autohydrolyzed lignin, which is a polymer with similar basic structure as the diphenolic lignans, protects against experimental colon adenocarcinoma in rats [158]. Lignin is also known to bind deoxycholic acid very well compared to other types of fiber [159]. The effect of rye bread (200 – 300 g per day, no other cereal products consumed) on intestinal bile acid metabolism was remarkable because it considerable decreased the total free bile acid, and total and free secondary bile acid concentrations and the ratio of secondary to primary bile acids in feces (J. T. Korpela, H. Adlercreutz & R. Korpela, to be published) leaving, however, the LCA/DCA ratio unchanged. This ratio increased with consumption of the fiber-free wheat bread. The reason for the decrease in free bile acids was a huge increase in the concentration of saponifiable (esterified) bile acids to a mean of about 46 % of total bile acids. These esters have been found to form a high proportion of the bile acids in feces in vegetarians (up to 80 %) but occur in very low amounts in CC patients (mean about 10 % of total bile acids) [160]. According to our theory the saponifiable (esterified) bile acids may not be cocarcinogenic or comutagenic as found for the aminoconjugates of these acids [16]. The reason for this may be that they are nonpolar and therefore less water-soluble which may be advantageous [152]. With the other types of bread practically no change of bile acid pattern occurred, or if any, it was in the opposite direction, particularly with respect to the fiber-free wheat bread. This is in agreement with a previous study showing no change in fecal bile acid excretion after consumption of a wholemeal bread compared to "white bread" [161]. In this connection it is of interest to note that during wartime the milling of flour resulted in much higher fiber, and possibly lignan precursor contents, which seems to have resulted in a modest decrease in colon cancer mortality [162]. These results would imply that by a simple change of the bread consumption to a daily intake of 200 – 300 g of whole-grain rye bread (or some other grain?), containing all the components of the cereal, the risk for both BC and CC could at least theoretically be reduced. Interestingly recent associations have been found both between BC [163] and CHD [164], and adenomatous polypes in colon, which are regarded as the first stage of some CC tumors.

Our results with respect to fecal bile acid metabolism are not in disagreement with the original theory of Hill *et al.* [13], but extend the theory to include the degree of "esterification" (the saponifiable bile acids have not yet been characterized). It is still most likely that the concentration of free secondary bile acids is an important factor determining the CC risk [15–20, 165, 166].

CONCLUSIONS

In conclusion, it seems that a Western diet with high fat and protein intake and low intake of fiber, complex carbohydrates and whole-grain products is associated with high plasma sex hormone levels and low SHBG, 5α-DHT, high %FT and %FE2, high urinary and low fecal excretion of estrogens, high urinary catecholestrogens excretion and 2-hydroxy-E1/4-hydroxy-E1 ratio, and low urinary excretion of lignans and isoflavonic phytoestrogens. These compounds apparently are protective with regard to cancer by many different mechanism. With respect to plasma hormones (except 5α-DHT), urinary lignans and equol we found this pattern in the postmenopausal BC women in Boston. Furthermore such a diet leads to unfavourable plasma lipid levels and intestinal bile acid metabolism most likely increasing the risk for both CHD and CC. In the study in Finland, where the BC and CC incidences are much lower than in USA, the hormonal pattern in the young BC patients was very similar to that of the control omnivorous and vegetarian women (33, 34), probably because of the relatively high intake of grain products [41] in all groups studied, but mean grain intake was still lowest in the BC group. The situation may be different in premenopausal compared to postmenopausal women, but still nothing speaks against the theory that diet is an important BC risk factor. This seems to be the fact particularly in the postmenopausal women, but probably and perhaps to a lesser degree, also in young women. All dietary components seem to have their specific role(s) in influencing sex hormone metabolism as described above and in this way a wrong diet may influence the development of BC and other sex hormone-dependent cancers in the promotional stage of the disease. More work is still needed, but already now it seems that the above-mentioned studies showing very distinct associations between diet and sex hormones and SHBG and diet and fecal bile acid pattern fit rather well with the view of the epidemiologists, that Western diet is the main factor causing the high incidence of hormone dependent cancers and CC in the Western world. Furthermore, many significant biochemical and hormonal connections between BC and other Western diseases, like CHD, exist, indicating that the same type of diet partly by the same mechanisms may be responsible for several of these diseases.

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D15

Revised & Updated

SUSAN M. LARK, M.D.

The Menopause SELF HELP BOOK

A Woman's Guide to Feeling Wonderful for the Second Half of Her Life

The first completely practical, all-natural guide for relief and control of every symptom of menopause

Dr. Susan Lark's

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FOR THE SECOND HALF OF HER LIFE

Susan M. Lark, M.D.

The first completely practical all-natural master plan to relieve and prevent every symptom of menopause

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CELESTIALARTS
Berkeley, California

To my wonderful husband Jim, thank you for all your help. And to my darling daughter Rebecca, thanks for being so much fun.

READER PLEASE NOTE: The information in this book is meant to complement the advice and guidance of your physician, not to replace it. If you are under the care of a physician, you should discuss any major changes in your regimen with him or her. Because this is a book and not a medical consultation, keep in mind that the information presented here may not apply in your particular case. Whenever a question arises, discuss it with your physician.

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Berkeley, California 94707

Cover design by Ken Scott
Photographs by Pete Macchia
Composition by Wilsted & Taylor
Illustrations © 1990 by Ellen Joy Sasaki

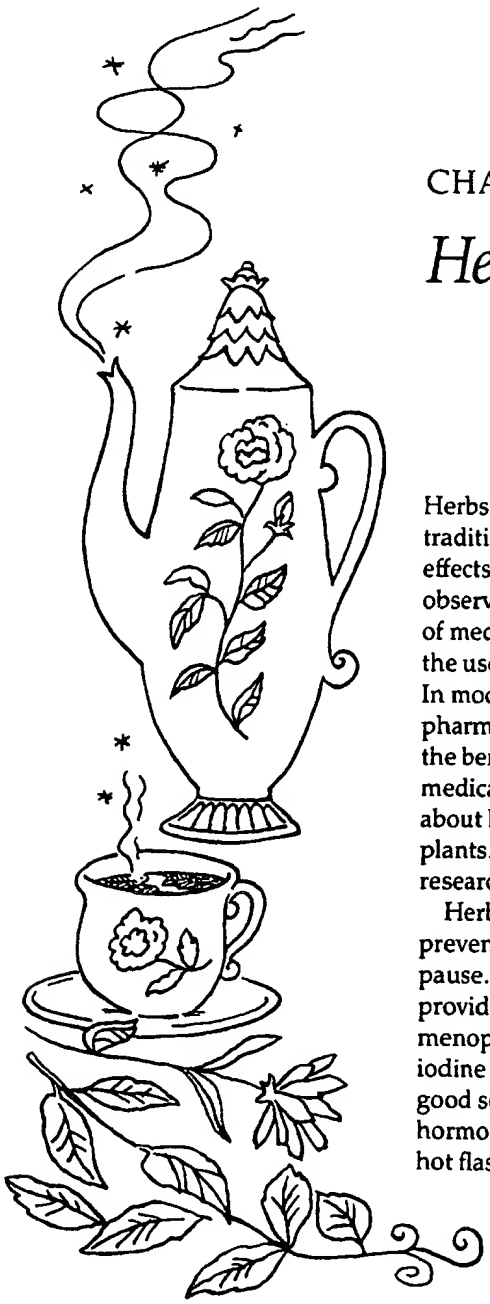
Library of Congress Cataloging-in-Publication Data
Lark, Susan M., 1945-
The menopause self help book / Susan M. Lark.
p.224 cm
Includes bibliographical references.
ISBN 0-89087-592-8
1. Menopause—Popular works. 2. Middle aged women—Health and hygiene. I. Title.
RG186.L37 1990
618.1'75—dc20 89-25292 CIP

First Printing, 1990

6 7 8 9 10 — 96 95 94 93

Manufactured in the United States of America

Contents



CHAPTER 10

Herbs for Menopause

Herbs were humankind's first medicine and formed the basis of traditional healing practices for thousands of years. Their benficial effects were discovered slowly through trial and error. By careful observation, early cultures learned to recognize the healing effects of medicinal plants for a variety of illnesses and learned to avoid the use of others because of their poisonous or harmful side effects. In modern times many research studies in the fields of botany, pharmacology, and medicine have allowed us to better understand the beneficial effects of many plant substances. Many interesting medical studies have shown that the traditional body of knowledge about herbs was correct in assigning healing properties to many plants. (See the bibliography at the end of this chapter for a list of research studies on herbs.)

Herbs can be a very useful part of your nutritional program to prevent or help balance a variety of symptoms related to menopause. They should be thought of as a form of extended nutrition, providing many nutrients that are necessary for good health in the menopause years. For example, dulse and kelp provide valuable iodine for optimal thyroid function, and red raspberry leaves are a good source of calcium and magnesium. Plants also contain natural hormones and a variety of substances that help to control bleeding, hot flashes, anxiety, insomnia, and other common menopause

symptoms. In the following section I will give information on specific herbs that can help relieve menopause symptoms.

Herbs for Your Menopause Symptoms

Heavy irregular menstrual bleeding. Plants that contain bioflavonoids help to strengthen capillaries and prevent heavy, irregular menstrual bleeding (menorrhagia). This is a common bleeding pattern as women approach menopause. Flavonoids are found in a large variety of fruits and flowers and are responsible for their color. Excellent sources are citrus fruits, cherry, grape, and hawthorn berry. According to research studies, they have also been found in red clover and subterranean clover strains in Australia. Many medical studies of citrus bioflavonoids have demonstrated their usefulness in a variety of bleeding problems, besides those related to menopause, such as habitual spontaneous abortion and tuberculosis.

Hot flashes. Many plants are good sources of estrogen, the hormone that helps to control hot flashes. Besides controlling heavy menstrual bleeding, bioflavonoids also have weak estrogenic activity (1/50,000 the strength of estrogen). They are very effective in controlling such common menopause symptoms as hot flashes, anxiety, and irritability. Plants containing bioflavonoids may be particularly useful for women who cannot take supplements because of their concern with the strong side effects of the prescription hormones (increased risk of stroke, cancer, etc.). Other plants sources of estrogen and progesterone used in traditional herbology include dong quai, black cohosh, blue cohosh, unicorn root, false unicorn root, fennel, anise, sarsaparilla, and wild yam root. The hormonal activities of these plants have been studied in a number of interesting research studies.

Plants may also form the basis for the production of medical hormones. Many common plants such as soy beans and yams contain a preformed steroidal nucleus. Estrogen and progesterone can be synthesized from plants in relatively few steps and have allowed

sex hormones to become available commercially at a reasonable cost.

Menopause anxiety, irritability, and insomnia. Women with menopause anxiety, irritability, and insomnia have a number of herbal remedies to choose from for relief of their symptoms. Herbs such as passionflower and valerian root have a significant calming and restful effect on the central nervous system. Passionflower has been found to elevate levels of the neurotransmitter serotonin. Serotonin is synthesized from tryptophan, an essential amino acid that has been found in numerous medical studies to initiate sleep and decrease awakening. Valerian root has been used extensively in traditional herbology as a sleep inducer. It is used widely in Europe as an effective treatment for insomnia. Research studies have confirmed both the sedative effect of valerian root and its effectiveness as a means to treat insomnia. For women with menopause insomnia, valerian root can be a real blessing. I have used it with patients for the past fourteen years and noted much symptom relief. Other effective herbal treatments include camomile, hops, catnip, and peppermint teas. I have used all of them in my practice and many pleased patients have commented on their effectiveness.

Menopause fatigue and depression. For women with menopause fatigue and depression, herbs such as oat straw, ginger, cayenne pepper, dandelion root, siberian ginseng (eleutherococcus), and blessed thistle may have a stimulatory effect, improving energy and vitality. Women who use these herbs may note an increased ability to handle stress, as well as improved physical and mental capabilities. Some of the salutory effects may be due to the high levels of the essential nutrients contained in herbs. For example, dandelion root contains magnesium, potassium, and vitamin E, while cayenne has high levels of magnesium and bioflavonoids. These are essential nutrients that have been found to help relieve menopause fatigue, depression, and hot flashes in a number of research studies. Siberian ginseng, ginger, and licorice root have been important traditional medicines in China and other countries for thousands of years. They have been reputed to increase longev-

ity and decrease fatigue and weakness. These herbs have been used to boost immunity and to strengthen the cardiovascular system. In modern China, Japan, and other countries there has been much interest in the pharmacological effects of these traditional herbs. Scientific studies are corroborating the important medicinal effects of these plants. Oat straw has been found in research studies to relieve fatigue and weakness, particularly when there is an emotional component.

Menopause urinary tract symptoms. Many herbs appear to have an ability to soothe, relieve irritation and reduce infection in the urinary tract, including goldenseal, uva ursi, blackberry root, and wintergreen. Research studies suggest that the plant coleus forskohlii also decreases urinary tract pain and discomfort. The urinary tract is a particularly vulnerable area in women during the menopause years and beyond because the lack of hormonal support causes the tissues to become more delicate and easily traumatized. Goldenseal contains berberine, an alkaloid with antibiotic activity, while uva ursi contains arbutin, a urinary diuretic and anti-infective agent. Coleus forskohlii contains forskolin, an antispasmodic which can relieve painful urination as well as menstrual cramps and intestinal colic.

Menopause and sexual frigidity. Herbs have also been popular treatments for the relief of sexual frigidity and impotence. Many cultures hold certain plants in high esteem for their aphrodisiac properties. On closer inspection, some of these plants, like spanish fly or nutmeg, have been found to be genitourinary irritants, rather than sexual stimulants. Traditional Indian medicine considers a number of plants such as saffron crocus and priya-darsa to have extraordinary aphrodisiac powers. Yohimbe, a plant aphrodisiac, is the base of several drugs currently prescribed to treat impotence.

Menopause Herbal Formulas

I have used herbs in my medical practice for years as a form of extended nutrition for menopause. They are an effective means of

balancing the diet and optimizing the nutritional intake. There are three herbal formulas that I use to provide optimal nutritional support for women suffering from menopause-related complaints. Formula I can be used by women with general menopause complaints such as hot flashes and vaginal dryness due to hormonal deficiency. Formula II is very helpful for women with menopause-related fatigue, debility, and weakness. Formula III can be used by women with menopause-related anxiety, irritability, and insomnia. Formula I is the basic herbal formula for menopausal women. Formulas II and III should also be used if you have the symptoms for which are applicable.

Herbal Formula I can be ordered by mail through the Menopause Self Help Center (see coupon at the back of the book). Formula I is also widely available in health food stores through Schiff Products.

Herbal Formula I:	Blue Cohosh
	False Unicorn Root
	Fennel
	Anise
	Blessed Thistle
Herbal Formula II:	Ginger
	Cayenne Pepper
	Siberian Ginseng (eleutherococcus)
Herbal Formula III:	Valerian root
	Catnip
	Camomile
	Hops
	Red raspberry leaf

The herbs should be used in small amounts and taken with your meals either in capsule form or in a tea. If you prefer to make a tea, simply empty the capsule into a cup of boiling water and let it steep for a few minutes. Do not drink more than one or two cups of the tea per day.

All foods have the potential for causing distress in some people,

and herbs are no exception. They should be discontinued immediately if you notice nausea, vomiting, or diarrhea upon using. These are the most common symptoms of intolerance. The herbs in my formulas are all recommended as being safe for human consumption, but some women seem to have a specific intolerance for various foods, including herbs. If you notice any symptoms that make you uncomfortable after using the herbs, discontinue them immediately.

Herbs for Menopause and Female Health Problems

Symptoms	Herbal Treatments
Menorrhagia	Shepherd's purse Hawthorn berry Cherry Grape skin Bilberry Red clover
Menopause hot flashes, Vasomotor symptoms	Dong quai Black cohosh Blue cohosh Unicorn root False unicorn root Fennel Sarsaparilla Red clover Wild yam root
Menopause insomnia and anxiety	Valerian root Passion flower Peppermint Catnip Camomile Hops

Menopause fatigue, tiredness, and depression	Oat straw Ginger Cayenne pepper Dandelion root Siberian ginseng Blessed thistle
Menopause bladder and lower urinary tract symptoms	Coleus forskohlii (pain) Goldenseal (infections) Uva ursi (infections) Blackberry root (infections) Wintergreen
Osteoporosis	Red raspberry leaf Comfrey
Hypothyroidism	Irish moss Kelp Dulse Sarsaparilla
Breast lumps and tenderness	Alfalfa Kelp Poke root poultices

Plants Used as Starting Materials for Commercial Hormone Synthesis

Plant Source	Preformed Steroidal Nucleus
Soybean	Stigmasterol
Calabas bean	Stigmasterol
Yeast	Ergosterol
Cereal grains	B-Sitosterol
Yams	Diosgenin
Sisal	Hecogenin

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D16

Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet[1-4]

Herman Adlercreutz, Hideo Honjo, Akane Higashi, Theodore Fotsis, Esa Hämäläinen, Takeshi Hasegawa, and Hiroji Okada

ABSTRACT Epidemiologic studies revealed low mortality in hormone-dependent cancer in Japanese women and men consuming a traditional diet. We previously found that certain diphenolic food components, lignans and isoflavonoids, which are converted to biologically active hormone-like substances by intestinal microflora, may be cancer-protective agents. Therefore, we studied urinary excretion of these compounds (enterolactone, enterodiol, daidzein, equol, and *O*-desmethylangolensin) in 10 women and 9 men in a rural village south of Kyoto, Japan. The subjects consumed a typical low-fat diet with much rice and soy products, fish, and vegetables. An isotope-dilution gas chromatographic-mass spectrometric method was used for the assays. The urinary excretion of lignans was low but that of the isoflavonoids was very high. The excretion of isoflavonoids correlated with soybean-product intake. The low mortality in breast and prostate cancer of Japanese women and men, respectively, may be due to the high intake of soybean products. *Am J Clin Nutr* 1991;54:1093-1100.

KEY WORDS Japanese, diet, urine, lignans, isoflavonoids, enterolactone, enterodiol, daidzein, equol, genistein, *O*-desmethylangolensin, soybean, gas chromatography, mass spectrometry, sex-hormone-binding globulin

Introduction

Mammalian lignans and isoflavonoid phytoestrogens, occurring in all studied animal and human biological fluids and in feces, are diphenolic compounds with molecular weights similar to those of steroid estrogens (1-3). Precursors in plants seem to occur as glycosides (4, 5), and the mammalian compounds are produced from plant lignans and isoflavonoids by intestinal microflora (6-8). Most of the original plant aglycones, such as formononetin, matairesinol, and secoisolariciresinol, occur only in very low concentrations in urine (9, 10). All compounds investigated so far are weakly estrogenic but have shown many other biological activities, producing antiestrogenic (1-3); antiviral (11, 12); and antiproliferative, cytotoxic, and growth-inhibiting effects (3, 13-15). Studies indicate that they most likely stimulate the production of sex-hormone-binding globulin (SHBG) in the liver (2, 14-18) and may in this way significantly influence biological activity of the sex hormones. The higher SHBG values seen in vegetarians (2, 17-19) are probably due to the effect of these diphenolic compounds on liver synthesis of the protein (14). Studies in both young and old women with breast cancer and in various dietary groups indicate that urinary excretion of these compounds is highest in vegetarians and lower in omnivores and breast-cancer patients (2, 18, 20). It was shown that their urinary excretion correlates with the intake of fiber-rich food (2, 17, 18).

Japanese women and women of Japanese origin in Hawaii consuming a diet similar to the original traditional Japanese diet have low breast-cancer incidence and mortality (21-24). Similarly, Japanese men have low mortality with prostate cancer, although autopsy studies have found that the incidence of prostate cancer in Japanese and Western men are similar (25-27). These cancers are sex-hormone dependent and could potentially be influenced both by alterations of sex-hormone metabolism caused by lignans and isoflavonoids or by a direct effect of these compounds on their growth. Because of the associations between diet and these diseases, we decided to study the urinary excretion of lignans and isoflavonoid phytoestrogens in groups of Japanese men and women consuming a traditional diet. A preliminary report was published as an abstract (28).

Subjects and methods

Participants

The subjects participating in this investigation were apparently healthy and were recruited in a small rural village south of Kyoto,

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[2] Preliminary report published as an abstract.

[3] Supported by Sigrid Jusélius and Finnish Cancer Foundations and the Medical Research Council of the Academy of Finland.

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Received January 4, 1991.

Accepted for publication April 17, 1991.

Japan. Two of the women were found to have hypertension (blood pressure 146/96 and 180/100, respectively). Most of the participants were farmers cultivating tea and rice. Originally 10 men and 10 women volunteered for the study, but 1 man was dropped because his urine volume was not known. Their main work was in agriculture and they consumed mainly their own products. The ages of the men and women were 50.4 ± 18.0 and 46.8 ± 11.5 y, respectively. Height, weight, and body mass index [BMI, in weight (kg)/height $(m)^2$] were, respectively, 160.8 ± 7.8 cm, 58.6 ± 5.8 kg, and 22.7 ± 2.3 for men and 153.1 ± 6.5 cm, 52.9 ± 7.2 kg, and 22.6 ± 3.5 for women. All subjects were within 15% of normal weight.

Collection of samples

Urine was collected for 48 h in plastic bottles containing 2 g ascorbic acid. The bottle was kept in a cool place during collection. The urine was mixed and measured and a sample was frozen as soon as possible and transported to Finland in dry ice for analysis.

Dietary data

The study was carried out in October 1985. Before the survey a nutritionist explained how to weigh the food components and how to write down the results on a form. Most of the food was weighed. Some food, such as bread and milk, was recorded as a piece of bread or cup of milk and the nutritionist estimated the weight of these food items afterwards. Food intake was recorded for 3 d and the nutritionist followed all subjects every day during the survey period. Calculation of the food data was made by an experienced nutritionist using the *Standard Tables of Food Composition in Japan* (29); for fiber calculations the *Food Composition Tables of Dietary Fibers, Minerals, Cholesterol, Fatty Acids* was used (30). The amount of soy sauce in the diet was calculated from the total sodium chloride content of the urine. According to earlier studies Japanese obtain 25.8% of their sodium chloride from soy sauce (31). Soy sauce contains 15% NaCl. The consumption of soy sauce is estimated by using the following formula:

$$\text{Soy sauce} = (\text{amount of NaCl in urine}) \times 0.258/0.15$$

This is the traditional way to estimate soy sauce consumption in Japanese subjects because they do not add any other salt to their food. It is an estimate and not an exact figure and the values were not included in the correlation analyses.

Analytical method

The trivial and systematic names of the compounds measured and discussed are as follows [structures were shown previously (3)]: enterolactone (Enl), *trans*- 2,3-bis[(3-hydroxyphenyl)methyl]-γ-butyrolactone; enterodiol (End), 2,3-*bis*[(3-hydroxyphenyl)-methyl]-butane-1,4-diol; daidzein (Da), 4',7-dihydroxyisoflavone; equol (Eq), 4',7-dihydroxyisoflavan; *O*-desmethylangolensin (*O*-Dma), 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-propan-1-one.

The method used was a modification of a method for determining the estrogen profile in urine by ion-exchange chromatography and capillary gas chromatography–mass spectrometry in the selected ion-monitoring mode (GC-MS-SIM, or GC/MS) (32–34). Originally, estrogens also were determined but because of very low concentrations of some fractions, the amount of urine saved for the purpose was too small and the analyses could not be repeated. Therefore, only the lignan and isoflavonoid values are presented. Only modifications of the method are described.

Protection of the carbonyl functions by ethoximation (necessary only for the estrogens), and extraction with a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) were carried out as described (33, 34). The removal of inhibitors of the enzyme hydrolysis by ion-exchange chromatography on a DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) column in the acetate form was done in a smaller column (0.5 × 3 cm instead of 0.5 × 5 cm). For hydrolysis and purification of the hydrolysate, before evaporation of the last fraction obtained from the above DEAE-Sephadex column, the following deuterated internal standards were added to the eluate: d_6-Enl and -End, d_4-Da and -Eq, and d_5-*O*-Dma (35, 36). This was followed by hydrolysis and Sep-Pak extraction; application of the methanolic extract directly on the QAE-Sephadex A-25 in the acetate form (0.5 × 5 cm); and elution of the estrogens, lignans, and Eq with 4 mL methanol as described. The modification in this step is that *O*-Dma and Da are eluted after this with 4 mL 0.2 mol acetic acid/L in methanol. This fraction is then, after evaporation of the solvent, ready for derivatization (trimethylsilyl ethers) and GC/MS. Selective fractionation of estrogens with vicinal *cis*-hydroxyls was carried out in a borate column with new dimension (0.5 × 3 cm instead of 0.5 × 2.5 cm). Elution of the diphenols was carried out as described and this fraction contains the isoflavan Eq and the two lignans Ent and End.

The two fractions containing lignans and isoflavonoid phytoestrogens and their deuterated internal standards are converted to their trimethylsilyl ether (TMS) derivatives (32) and quantified by GC/MS by using the following ion pairs (mass/charge): Eq, 386/390; Da, 398/402 (and 383/387); End, 410/416; Enl, 442/448; and *O*-Dma, 459/464 (36). The measurements were carried out with a Hewlett-Packard 5995 B GC/MS (Avondale, PA) instrument equipped with a Pascal work station and with an automatic injector.

Urinary excretion of < 0.0025 μmol/d cannot be measured, and between 0.0025 and 0.005 μmol/d the method must be regarded as semiquantitative. The mean values and interassay imprecision for the control pooled-urine sample, measured 59 times in single assays during 1 y, were as follows: Enl, 3.65 μmol/d (CV 7.4%); End, 0.364 μmol/d (CV 11.6%); and Eq, 0.042 μmol/d (CV 9.4%). For Da at a concentration of 0.028 μmol/d, the interassay imprecision is 11.0% (n = 14) and for *O*-Dma at the high concentrations in this study, the interassay imprecision is 8–10% (CV).

The samples were analyzed in two batches and the values for the control sample were almost identical both times and the same as in analyses before and after these two batches.

Statistical methods

The food data are presented as arithmetic means (±SD) and the lignan and phytoestrogen results as arithmetic means (±SD) and geometric means. Geometric means were used when necessary because of skewness of the distribution of the results. The statistical analyses were carried out by using the *StatView* program for Macintosh (Abacus Concepts, Berkeley, CA). The degree of univariate associations between two variables were estimated as Pearson's correlation coefficients (r). The pairs of

TABLE 1
Intake of various food stuffs by the Japanese women and men consuming a traditional Japanese diet*

Nutrient	Women (n = 10)	Men (n = 9)
	g/d	
Rice	578.5 ± 222.5	764.7 ± 240.3
Wheat	59.5 ± 46.0	139.0 ± 113.6
Potato	62.6 ± 30.2	55.2 ± 34.6
Sugar	8.1 ± 7.0	8.1 ± 7.4
Fats	13.1 ± 7.6	12.7 ± 6.9
Pulses and beans	56.5 ± 36.0	40.9 ± 32.0
Fruit	228.2 ± 111.9	146.9 ± 114.0
Green and yellow vegetables	60.6 ± 33.3	55.7 ± 35.2
Other vegetables	139.3 ± 69.3	130.9 ± 77.2
Pickles	32.9 ± 24.9	23.2 ± 21.2
Algae	1.8 ± 2.0	0.7 ± 0.7
Fish	98.7 ± 46.6	113.6 ± 56.5
Meat	37.0 ± 30.1	73.6 ± 58.4
Eggs	38.4 ± 16.6	57.4 ± 30.6
Milk	112.7 ± 131.0	90.9 ± 90.2
Beer	5.1 ± 16.1	454.6 ± 647.1

* $\bar{x}$ ± SD.

adjusted group means for the two groups studied (women and men) were compared by nonpaired t test.

Results

The intake of various types of food are shown in **Table 1**, and Table 2 shows the results of the calculations with regard to energy;

TABLE 2
Energy intake, intake of various nutrients, and some ratios in the two study groups*

Nutrient	Women (n = 10)	Men (n = 9)
Energy		
(MJ/d)	8.29 ± 1.64	10.79 ± 3.48
(kcal/d)	1973 ± 391	2569 ± 829
Animal protein (g/d)	35.3 ± 13.9	47.8 ± 18.9
Vegetable protein (g/d)	38.2 ± 10.1	45.1 ± 10.6
Total protein (g/d)	73.6 ± 12.2	93.0 ± 28.4
Carbohydrates (g/d)	311.4 ± 77.0	383.3 ± 100.6
Total fat (g/d)	44.4 ± 14.4	51.0 ± 25.9
Total fiber (g/d)	16.9 ± 4.9	15.3 ± 6.0
Animal protein (%)†	47.2 ± 15.9	49.8 ± 7.9
Proteins (%)‡	15.2 ± 2.1	14.6 ± 1.5
Carbohydrates (%)‡	64.6 ± 6.8	68.2 ± 5.1
Fats (%)‡	20.3 ± 5.5	17.2 ± 4.9
Fat (g/kg body wt)	0.86 ± 0.31	0.85 ± 0.37
Fiber		
(mg/J)	2.1 ± 0.7	1.5 ± 0.7
(g/1000 kcal)	8.8 ± 3.0	6.4 ± 3.0
Fiber (g/kg body wt)	0.33 ± 0.10	0.26 ± 0.09
Fat-fiber ratio	2.5 ± 0.9	2.4 ± 0.9

* $\bar{x}$ ± SD.
† Percent of total protein.
‡ Percent of energy.

TABLE 3
Dietary intake of soy products by the two groups studied*

Soy product	Women (n = 10)	Men (n = 9
	g/d	
Tofu (soybean curd)	25.0 ± 22.9	18.7 ± 2
Miso (bean paste)	12.5 ± 6.2	8.5 ± 6.
Aburaage (fried thin tofu)	2.6 ± 3.6	3.7 ± 4.
Atuage (fried thick tofu)	4.0 ± 12.7	0.8 ± 2.
Koridofu (dried soybean curd)	0.37 ± 0.78	0.07 ± 0.
Fermented soybeans	2.4 ± 4.5	0.9 ± 2.
Boiled beans	7.7 ± 17.8	6.5 ± 7.
Soy sauce	22.9 ± 6.1	19.2 ± 4.
Soy products (sauce excluded)	54.4 ± 34.3	39.2 ± 3(

* $\bar{x}$ ± SD.

animal and vegetable protein; total proteins, carbohydrates, fa and fiber; percentage animal protein and percentage protei and carbohydrate and fat as percent of total calories. Furthe more, we calculated the fat intake per kilogram body weigl fiber intake per J (per 1000 kcal), and the fat-fiber ratio (Tab 2). The diet was a low-fat (fat 17.2% and 20.3% of total calori for men and women, respectively), low-animal-protein diet wi moderate amounts of fiber and a low fat-fiber ratio, which typical for the traditional Japanese diet (37).

Table 3 shows the dietary intake of soy products, which we expected to be the most important source of precursors for tl urinary isoflavonoids (3).

Table 4 shows the mean excretion values for the two ligna and three isoflavonoid phytoestrogens. The results show a re atively low excretion of enterolactone, a normal excretion f enterodiol, and a very high excretion of isoflavonoid phytoe trogens. The individual results showed large variation, partic larly for equol (from 0 to 10.95 μmol/d). For comparison no that the geometric mean values in young omnivorous wome living in Helsinki and in Boston for enterolactone, enterodic daidzein, equol, and *O*-desmethyl-angolensin were 2.46, 0.2 0.22, 0.10, 0.03, and 2.05, 0.28, 0.32, 0.07, and 0.03 μmol/ respectively (2).

TABLE 4
Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese women and men consuming traditional Japanese diet*

Compound	Women (n = 10)	Men (n = 9)
	μmol/d	
Enterolactone	1.4 ± 1.4 (0.89)	1.1 ± 0.7 (0.8
Enterodiol	0.7 ± 1.3 (0.41)	0.4 ± 0.3 (0.2
Total lignans	2.1 ± 2.6 (1.38)	1.5 ± 0.9 (1.1
Daidzein	2.6 ± 4.0 (2.55)	2.2 ± 2.0 (1.4
Equol	2.6 ± 4.0 (0.56)	3.0 ± 4.6 (0.5
O-desmethylangolensin	0.7 ± 0.6 (0.51)	0.2 ± 0.3 (0.1
Total isoflavonoids	6.9 ± 6.8 (4.73)	3.9 ± 3.3 (2.5
Total diphenols	9.1 ± 9.3 (6.7)	5.4 ± 4.0 (4.1)

* $\bar{x}$ ± SD (geometric $\tilde{x}$).

Table 5 presents a correlation matrix of various food components and urinary excretion of lignans and isoflavonoids in the total material of 19 subjects for whom both food and phytoestrogen data were available.

Discussion

In a previous study of oriental immigrant women from southeast Asia residing in Hawaii (38), the diet was similar to that consumed by the men and women in the rural village in Japan. In the present study the women had a greater energy intake (an additional ~2.1 MJ/d, or 500 kcal/d), which may be due to a physically more active life. However, the percentage intake of calories as fat and the dietary fiber and fat-fiber ratio were very similar to the corresponding values in the previous study. Except for the energy intake the values are very different from those seen in Western societies where the fiber intake is similar but the fat-fiber ratio is much higher. Women living in the Boston area had a fat-fiber ratio of 7.7 for the premenopausal women and 4.6 for the postmenopausal women compared with 2.5 for the women in the present study (39).

With regard to protein intake, expressed as g/d and as percentage of calories, the mean values in the present study were similar and slightly lower, respectively, than those of the immigrants from southwest Asia (38).

Our results are in good agreement with those from an earlier study of 300 female agricultural workers from 18 regions in Japan (37) except for dietary fiber intake, which was much lower (between 5 and 6 g/d) in the women in the earlier study (which may represent crude fiber intake). However, according to the national nutrition survey in Japan, the dietary fiber intake was 22.8 g/d in 1951 and decreased year by year to 17.4 g/d in 1985. These figures are in better agreement with our results obtained in 1985, which show a mean dietary fiber intake in the whole group of ~16 g/d. This latter value is also in good agreement with the value of 13 g/d for nonstarch polysaccharides found by analyses of the Japanese diet in another study (40). On the basis of these investigations and the present investigation, it may be concluded that the amount of dietary fiber in a traditional oriental diet is comparable with that in many Western societies (38–40). We may also conclude that the diet of our subjects was typical for a rural area, where the people to a large extent consume their own products and have a traditional Japanese diet.

The urinary excretion of Enl was, with few exceptions, low in both men and women (Tables 4 and 1A) and was the same as found for the postmenopausal breast-cancer patients in Boston (20). We found a weak correlation between intake of green and yellow vegetables and excretion of Enl and total lignans (Table 5) but no correlation with rice intake. Because these subjects consumed large amounts of rice, it seems justified to conclude that refined rice contains very low amounts, if any, of lignan precursors. There was a better correlation with the intake of soybeans, which thus also may be a source of Enl precursors (Table 5). It is known that soy sauce contains coniferyl alcohol the building block for lignans and lignin (41). The excretion of the lignan End was also found to be associated with the intake of beans and pulses and soy products in general (Table 5).

The excretion of the isoflavonoid phytoestrogens is very high in these Japanese men and women compared with values obtained in women living in Boston (2, 20) and in the Helsinki area (2, 18). The Japanese women in the present study excrete 10 times more Da and 20–30 times more Eq and *O*-Dma than did omnivorous and lactovegetarian women living in the above-mentioned two cities. Of the 19 subjects, 47% and 89% excrete micromole amounts of Eq and Da per day, respectively, a phenomenon very rarely seen in subjects consuming a Western diet but seen in subjects consuming a macrobiotic diet (2). The values in an additional study group of nine subjects, including three children (*see* Appendix A), were not significantly different from those in the two main groups (Tables 4 and 1A); they were in fact surprisingly identical. The excretion of matairesinol, the precursor lignan for enterodiol, was very low, but genistein excretion was very high. Genistein is the center of interest in many laboratories because of its very interesting antiproliferative and

TABLE 5
Correlation matrix of various food components and urinary excretion of ligans and isoflavonoids in the whole material (n = 19)

Nutrient	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Total isoflavonoids	Total diphenols
Green and yellow vegetables	0.525*		0.460*					
Pulses and beans		0.541*	0.492*	0.679†	0.737†	0.617†	0.668†	0.693†
Algae				0.561*			0.450‡	0.430‡
Total fat					0.584†			
Percent fat calories								
Fat-fiber ratio					0.469*			
Meat					0.507*			
Soy products (not sauce)		0.481*		0.583†	0.507*			
Boiled soybeans	0.758§	0.892§	0.849§	0.632†	0.746§	0.601†	0.585†	0.588†
					0.693§		0.757§	0.801§

* $P < 0.05$.
† $P < 0.01$.
‡ $0.05 < P < 0.10$.
§ $P < 0.001$.

antimitogenic effects (*see* below); genistein showed the highest concentration of all phytoestrogens in urine in these nine subjects. The mean value was almost 6 μmol/d and a value as high as 15.5 μmol/d was observed. Also in this smaller group most variation in the excretion values was found for Eq (from 0.01 to 9.16 μmol/d). In 21.4% of all subjects, equol excretion did not significantly differ from zero: this group included two of the three children; the mother of these two children did not excrete equol in significant amounts.

The low excretion of Enl in the Japanese subjects compared, eg, with Finnish women (2), is most likely due to low intake of grain (whole-grain) products such as bread (2, 17, 18, 42, 43). The precursors of the mammalian lignans seem to be located in the aleuronic layer of the grain close to the fiber (15) but definite evidence for this location has not yet been obtained. The mean End values are similar to those observed in lactovegetarian American and Finnish women and higher than in the omnivorous women from the same countries (2, 20). It is likely that the majority of the lignans in these Japanese subjects is derived from nongrain plant products (pulses and beans), as suggested by the correlations found in Table 5.

Eq excretion correlated positively with the intake of total fat ($P < 0.01$), fat-fiber ratio ($P < 0.05$), and meat ($P < 0.05$) and deviated in this aspect from all the other isoflavonoids. Some subjects are not able to produce Eq at all, as also shown previously for nonJapanese subjects (44). It is possible that those consuming more fat and meat have an intestinal flora more capable of producing Eq from Da, known to occur in large amounts in soybeans (45). Algae may also be a source of isoflavonoids because a positive correlation was found with Da ($r = 0.56$; $P < 0.05$) and total isoflavonoids ($r = 0.45$; $0.05 < P < 0.10$, NS). Algae were suggested to contain factors protective against breast cancer (46).

Lignans and bioflavonoids are candidates for a role as cancer-protective agents (2, 14–16) and as steroid competitors for various enzymes (47). Enl inhibits the aromatase enzyme and competes with the natural substrate androstenedione for the binding site on the cytochrome P450 enzyme (H Adlercreutz, C Bannwart, LE Vickery, et al, unpublished observations, 1985). Phytoestrogens and lignans (48; H Adlercreutz, Y Mousavi, J Clark, et al, unpublished observation, 1987) show interaction with estrogen receptors and flavonoids have antiproliferative effects on the human-breast-carcinoma cell line ZR-75-1 (49). Genistein is a very specific inhibitor of the tyrosine-specific protein kinases (50–55) and platelet-activating-factor–stimulated platelet aggregation, phospholipase C, and tyrosine kinase activity (56). Tyrosine kinase is an important mediator of the effects of some biologically important growth factors such as epidermal growth factor, insulin, platelet-derived growth factor, and insulin-like growth factor on cells. The flavonoids and lignans bind to the type II estrogen-binding sites (15, 57), now also called the bioflavonoid receptor (47, 58), and may in this way regulate by inhibition cell growth and proliferation of hormone-dependent cancers (58). Enzymes metabolizing bioflavonoids and steroids show structurally close similarity (47), indicating that they have the same origin. Furthermore, the isoflavonoid coumestrol complements, as does estradiol, the topography of spaces between base pairs in unwound DNA and simultaneously hydrogen-bond phosphate moieties on opposite strands (59).

One of the most important biological effects of the lignans and isoflavonoids seems to be their stimulation of SHBG synthesis in the liver (2, 14, 16–18). A high SHBG concentration leads to decreased metabolic clearance rate for the sex hormones and lower biological activity. However, Japanese and British women were found to have the same SHBG total-binding capacity, even though Japanese women bound relatively more estradiol to SHBG. This was suggested to be a result of lower affinity of albumin for estradiol in these women (60). It is possible that the phytoestrogens in the high amounts occurring in Japanese women could compete with estradiol for the albumin-binding sites and in this way lead to relatively more binding to SHBG.

SHBG concentrations tend to be lower in breast-cancer patients, particularly in postmenopausal women, and this seems at least partly to be due to diet (15). SHBG-binding capacity was significantly smaller in postmenopausal but not in premenopausal Japanese subjects with breast cancer compared with Japanese control subjects (61), agreeing with our own more recent results in American postmenopausal (43) women. Finnish premenopausal women with breast cancer did not differ in this respect from omnivorous control subjects but they had lower SHBG than did lactovegetarian women (18). Diet seems to be a much more important risk factor for postmenopausal than for premenopausal breast cancer (15). Miso (Japanese soybean paste) (62) or powdered soybean chips (63) (both before and after denaturation of the protease inhibitors) showed a tendency to decrease mammary-tumor formation and growth rate in rat breast-cancer models and soybean diet also reduced breast-tumor incidence in irradiated rats (64). This agrees with the slower average growth rate of postmenopausal breast cancers in Japanese compared with caucasian women in Hawaii (65).

The high concentration of phytoestrogens in the urine of Japanese men could be protective with regard to prostate cancer. Both lignans and isoflavonoids have estrogenic effects in numerous biological systems and may, because of this property, inhibit development of prostatic cancer. It is well known that in Japan and some other Asian countries, despite the same incidence of latent small or noninfiltrative prostatic carcinomas as in Western societies, the mortality is low (25–27). The high exogenous phytoestrogen concentrations could inhibit the growth of the latent carcinomas, postponing their development and making it more likely that the subjects die from some other disease (theory proposed in 1985) (66). Furthermore, the inhibitory effect of genistein on tyrosine-specific protein kinases of certain growth-factor receptors could play an important role. Decreased risk of prostate cancer is seen in Seventh-day Adventist men (67) consuming much beans, lentils, and peas and some dried fruits (rich sources of bioflavonoids) and in men of Japanese ancestry in Hawaii consuming much rice (mainly starch, which has some fiber-like effects in the gut) and tofu (68), supporting the view that these compounds are protective. Recently, Santti's group in Turku, Finland, in a collaborative study with us, observed that dietary soy prevented the development of precancerous changes in a neonatally estrogenized mouse used as a model for prostatic cancer (69), showing that dietary factors may already be important in the fetal and neonatal periods. This study and our observation of high phytoestrogen excretion in urine of children is important because they suggest that these compounds may change the endocrine milieu at the cellular level both in the neonatal period and in prepubertal and adolescent children. Thus, the results cited above and discussed more

extensively elsewhere (14, 15) speak for a role of the diphenols as cancer-protective substances.

It is concluded that Japanese subjects excrete very large amounts of isoflavonoids in urine, mainly genistein, daidzein, and equol, and that the lignan excretion is low. The high excretion of isoflavonoids in urine is related to the intake of soy products in the traditional Japanese diet.

We thank Anja Koskela (analytical work) and Sirkka Adlercreutz (mass spectrometry) for skillful technical assistance.

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APPENDIX A

Additional experiments with a modification of the method

The method used in this study was modified further by including the determination of the plant lignan matairesinol [(3R-*trans*)-dihydro-3,4-*bis*[(4-hydroxy-3-methoxy-phenyl)methyl]-2(3H)-furanone)] (intraassay CV = 15.2% and interassay CV = 13.9%) and the isoflavonoid genistein (4',5,7-trihydroxyisoflavane) (intraassay CV = 4.5% and interassay CV = 11.6%) in the assay (1). Because further samples from the present study were not available and because of the recent great interest in genistein we used this new assay in nine other Japanese subjects (three men, three women, and three children) living in Kyoto and consuming a traditional Japanese diet before and during the 24-h urine collection.

TABLE 1A
Urinary excretion of lignans and isoflavonoid phytoestrogens (μmol/d) in nine Japanese subjects (six adults, three children) living in Kyoto and consuming traditional Japanese diet during the urine collection period

Subject, sex, age	Matairesinol	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Genistein	Total isoflavonoids	Total diphenols
1, M, 41 y	0.010	0.05	0.09	0.15	5.25	6.15	0.12	15.52	27.04	27.20
2, F, 33 y	0.003	2.44	0.15	2.59	3.11	0.01	0.98	4.48	8.58	11.17
3, M, 7 y	0.003	0.07	0.09	0.16	3.23	0.01	0.06	5.66	8.97	9.13
4, M, 6 y	0.006	2.24	0.68	2.93	2.15	0.85	0.51	3.41	6.93	9.85
5, M, 8 y	0.007	0.04	3.39	3.43	3.02	0.02	0.81	4.80	8.64	12.07
6, F, 42 y	0.006	3.25	0.25	3.50	2.20	0.16	1.17	3.55	7.07	10.58
7, M, 38 y	0.012	0.70	0.25	0.96	1.60	0.07	0.40	4.93	6.99	7.95
8, M, 26 y	0.019	1.94	0.18	2.13	3.38	9.16	0.23	7.99	20.76	22.89
9, F, 30 y	0.005	0.62	0.25	0.88	1.25	3.28	0.21	1.85	6.60	7.47
$\bar{x}$	0.010	1.26	0.59	1.86	2.8	2.19	0.50	5.80	11.29	13.15
Geometric $\bar{x}$	0.010	0.50	0.27	1.17	2.58	0.25	0.35	4.91	9.81	11.89

Table 1A shows the individual urinary lignan and isoflavonoid excretion in the additional three men, three women, and three children studied by the new modified procedure, including the results of assays for matairesinol and genistein.

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Journal of Chromatography, 386 (1987) 315–323
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 956

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PHYTOESTROGENS IN SOY PROTEIN PREPARATIONS WITH ULTRAVIOLET, ELECTROCHEMICAL AND THERMOSPRAY MASS SPECTROMETRIC DETECTION

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SUMMARY

The phytoestrogens daidzein, genistein, coumestrol, formononetin, and Biochanin A are separated on a C_{18} reversed-phase column (Hypersil ODS) with methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (60:40, v/v) as eluent. The retention and resolution are affected by buffer concentrations, pH type, and proportion of organic solvent in the mobile phase. Detection in the (low pg range) is achieved with an electrochemical detector, and the compounds are positively identified by high-performance liquid chromatography–thermospray mass spectrometry. Daidzein and genistein were found in high concentrations in all soy protein preparations analyzed.

INTRODUCTION

The phytoestrogens are a group of naturally occurring plant products[1,2] possessing weak estrogenic activity[3–6]. Their existence in soybeans has been known for some time[7], and recently several phytoestrogens and their metabolites were identified in biological fluids of man[8–11]. In particular, the ingestion of soy protein has been shown to be associated with a vast increase in the urinary excretion of these compounds, and levels in vegetarians generally are higher than those for the general population[8,9,12]. Given the strong association between diet and disease[13], the potential implications, whether beneficial or deleterious, of ingesting biologically active compounds, such as phytoestrogens requires examination. This is particularly the case with the increasing use of soy-based products for human consumption[9], and important to such studies is the requirement of suitable techniques for the detection of these compounds in diets.

Methods for the separation of phytoestrogens in plant extracts by high-performance liquid chromatography (HPLC) have been described[14–22], however, these

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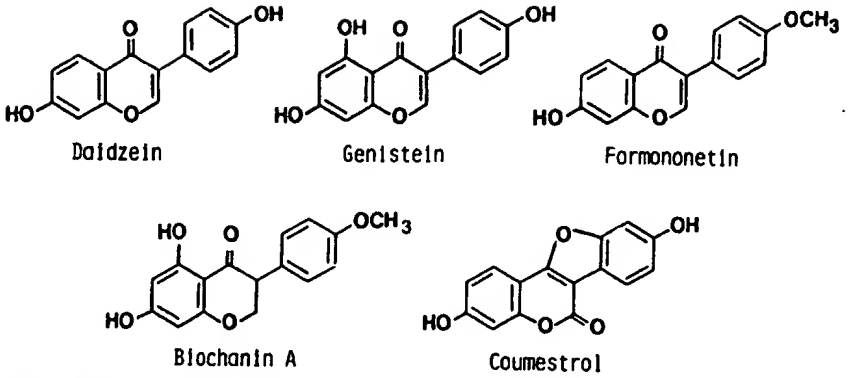


Fig. 1. Chemical structures of the principal plant phytoestrogens.

have generally used gradient elution systems. This paper describes a simple isocratic reversed-phase system, with methanol–0.1 *M* ammonium acetate, pH 4.6 (60:40, v/v) as mobile phase on an Hypersil ODS column, for the rapid and effective separation of the phytoestrogens daidzein, genistein, coumestrol, formononetin, and Biochanin A (Fig. 1). The effects that buffer concentrations, pH, and the type and concentration of organic solvent in the mobile phase have on the retention and resolution have been studied. The sensitivity of ultraviolet (UV) and electrochemical detection (ED) systems has been compared and conditions have been established for HPLC–thermospray mass spectrometry (MS) to allow the identity of individual phytoestrogens in the HPLC effluent to be confirmed. The method has been successfully applied to the analysis of phytoestrogens in a range of soybean products, including soy-based milk formulae and animal diets.

EXPERIMENTAL

Materials and reagents

Biochanin A, genistein, daidzein, and formononetin were from K & K Rare and Fine Chemicals (Plainview, NY, U.S.A.) and coumestrol from Kodak (Rochester, NY, U.S.A.). Textured soy and soy flakes were from Arrowhead Mills, Inc. (Heuford, TX, U.S.A.). Isomil was from Ross Laboratories (Columbus, OH, U.S.A.) and ProSobee was from Mead Johnson (Evansville, IN, U.S.A.). Ammonium acetate, glacial acetic acid, and EDTA were AnalaR-grade from BDH (Poole, U.K.). Acetonitrile and methanol were HPLC grade from Rathburn (Walkerburn, U.K.). The enzyme preparations, β-glucosidase and Helix pomatia (β-glucuronidase and sulfatase) were obtained from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

Samples of Isomil (50 ml), ProSobee (50 ml), or individually homogenized samples of textured soy (5 g) and soy flakes (5 g) were refluxed in 80% aq. ethanol (total volume 250 ml) for 2 h to extract all isoflavones, polar isoflavone conjugates, and related compounds. The organic extracts were cooled, centrifuged, and the supernatant was removed. The ethanol was evaporated in a rotary evaporator, and the lipids were extracted from the remaining aqueous extract by partitioning twice into

four volumes of hexane for textured soy and soy flakes, and partitioning four times for Isomil and ProSobee. The aqueous extract was taken to dryness. Hydrolysis of isoflavone conjugates was carried out using several enzyme preparations. The samples were first subjected to hydrolysis with a β-glucosidase preparation in 0.1 *M* acetate buffer (pH 5.0) overnight at 37°C. The hydrolysate was passed through a cartridge of reversed-phase octadecylsilane-bonded silica (Bond-Elut C_{18}; Analytichem, Harbor City, CA, U.S.A.) to extract all isoflavones, and after washing the cartridge with water, the isoflavones were recovered by elution with 5 ml methanol. After evaporation of the methanol to dryness a second hydrolysis was performed using 0.2 ml of a combined β-glucuronidase and sulfatase preparation (Helix pomatia) in 20 ml 0.5 *M* acetate buffer (pH 4.5) for 24 h at 37°C. The hydrolysate was again passed through a Bond-Elut cartridge to extract the isoflavones, which were recovered by elution with 5 ml methanol and taken to dryness under nitrogen on a 65°C heating block, and the residue was reconstituted prior to assay.

HPLC

A Varian (Walnut Creek, CA, U.S.A.) Model 5000 liquid chromatograph and a Varian UV-100 variable-wavelength detector or a LCA-15 electrochemical detector (EDT Research, London, UK) were used. The electrochemical detector was of the wall-jet type with a glassy-carbon working electrode and a Ag/AgCl reference electrode. Samples were injected via a Rheodyne 7125 injector (Cotati, CA, U.S.A.), fitted with a 100-μl loop.

The column (25 cm × 4.6 mm I.D.) was Hypersil ODS, 5 μm spherical silica, chemically bonded with a monolayer of octadecylsilyl groups from Shandon Southern Products (Runcorn, UK). The mobile phase was methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (60:40, v/v), containing 0.25 mmol/l EDTA. The mobile phase was continuously degassed with a stream of helium during ED. This is unnecessary for UV detection, and EDTA can also be omitted from the mobile phase. The flow-rate was 1 ml/min at ambient temperature and UV detection was at 260 nm. ED of phytoestrogens was achieved at different operating potentials in the range +0.4 to +1.2 V.

Mobile phases of different buffer concentrtions, pH, and with acetonitrile and acetonitrile–methanol mixtures as organic modifiers were used to study their influence on retention and resolution of the individual phytoestrogens.

HPLC–MS

The Varian Model 5000 high-performance liquid chromatograph was coupled to a Finnigan 4635 quadrupole mass spectrometer via a thermospray interface (Finnigan). The mass spectrometer was operated in continuous scanning mode over a mass range of 110–300 daltons. The optimum interface temperatures at the flow-rate used for HPLC separation of the phytoestrogens were determined by multiple injection of standards and varying heater temperatures and repeller voltages. Optimum conditions for the ionization of all the phytoestrogens studied were obtained with a vaporizer temperature of 135°C and a jet-block temperature of 215°C. Solvent flow-rate was 1.0 ml/min, and the HPLC conditions were as described above.

RESULTS AND DISCUSSION

HPLC

The separation of a standard mixture of phytoestrogens on Hypersil ODS with methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (60:40) as eluent is shown in Fig. 2. The elution of these compounds was in the order: daidzein, genistein, coumestrol, formononetin, and Biochanin A. Genistein with three phenolic groups (Fig. 1) is expected to be less hydrophobic than, and therefore eluted before, daidzein, having two phenolic groups. Similarly, Biochanin A with two phenolic groups should, under normal circumstances, be eluted before formononetin with only one phenolic group. The observed reversal in elution order is probably due to the ability of genistein and Biochanin A to form intramolecular hydrogen bonds between one of the phenolic group and the keto group, as shown in Fig. 3. Intramolecular hydrogen bonding will decrease the polarity (increase hydrophobicity) of the molecules, leading to longer retention[21].

Methanol is a better modifier than acetonitrile and is essential for the separation of genistein and coumestrol. The resolution of these two compounds is lost when methanol is replaced with acetonitrile as the modifier (Fig. 2). However, ternary systems which include methanol as one of the organic components will still resolve genistein and coumestrol. For example, a ternary system of acetonitrile–methanol–0.1 *M* ammonium acetate buffer, pH 4.6 (10:50:40) gave a resolution similar to that with the methanol–buffer system.

The pH and buffer concentration of the mobile phase affects the retention but not the resolution. Increasing the pH and/or the buffer concentration decreases the retention of all compounds while maintaining the resolution. A 0.1 *M* buffer at pH

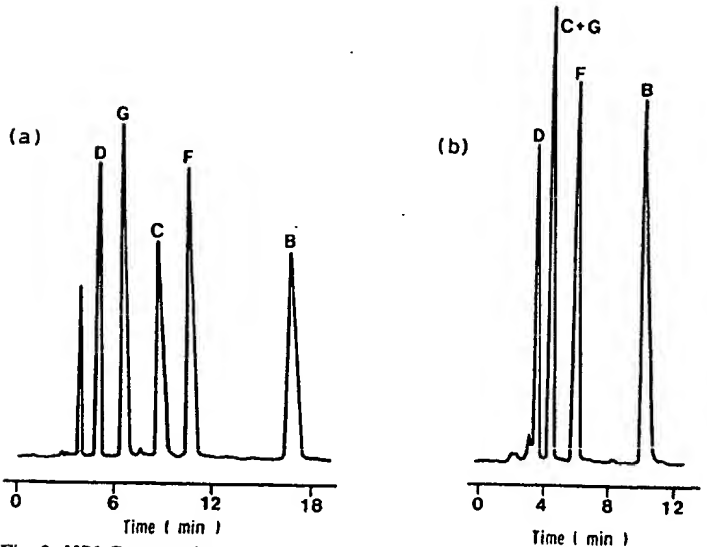


Fig. 2. HPLC separation of phytoestrogen standards, illustrating the effect of varying the mobile phase. (a) Mobile phase: methanol–0.1 *M* ammonium acetate, pH 4.6 (60:40); (b) acetonitrile–0.1 *M* ammonium acetate, pH 4.6 (47:53). Flow-rate 1 ml/min. 260 nm. The following compounds are indicated: D = daidzein; G = genistein; C = coumestrol; F = formononetin; B = Biochanin A.

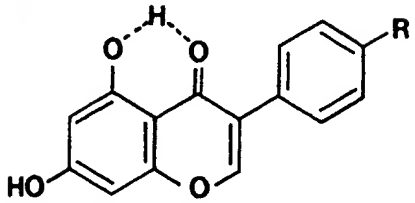


Fig. 3. Intramolecular hydrogen bonding between the phenol and keto groups in the structures of the phytoestrogens genistein (R = OH) and Biochanin A (R = OCH_3).

4.6 was chosen to provide rapid and yet adequate separation of the phytoestrogens, particularly of the early eluted peaks, from possible interferences in sample extracts.

Choice and sensitivity of detector

The phytoestrogens can be detected by UV absorption at 260–280 nm with a detection limit of about 5 ng injected (signal-to-noise ratio of 3 at 0.002 a.u.f.s.). The phytoestrogens are also electroactive, due to the presence of phenolic groups, and can therefore be detected with ED. Coumestrol is the most electroactive compound, followed by genistein and daidzein. The voltammagram for these three compounds is shown in Fig. 4. The optimum potential for the simultaneous sensitive detection of all three compounds is +0.75 V. At a detector sensitivity of 3 nA, the detection limits of coumestrol, genistein and daidzein are 5, 10, and 15 pg injected, respectively. Thus, ED is much more sensitive than the UV detector. However, the satisfactory ED of formononetin and Biochanin A required an operating potential above +1.2 V. At this detector potential, baseline stability becomes a problem. These two compounds are therefore better detected with an UV detector. An UV detector may also be coupled in series with an electrochemical detector for the detection of a wide range of phytoestrogens. However, in preparations containing only daidzein and genistein, ED is the obvious detection system of choice. It allows a much smaller sample size to be used, and therefore a simpler and a cleaner matrix is obtained. For the specific detection of coumestrol a lower operting potential (+0.45 to +0.5 V) may be used, since few compounds are electroactive at these low potentials.

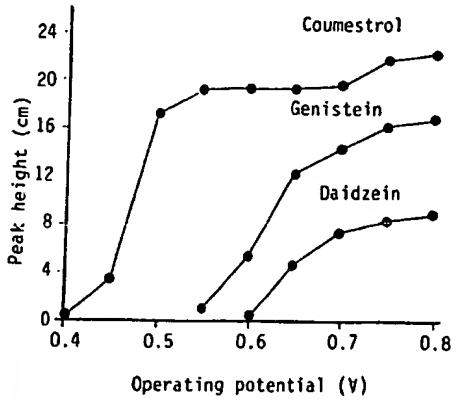


Fig. 4. Voltammagrams for the phytoestrogens coumestrol, genistein and daidzein.

HPLC–thermospray MS

With the development and introduction of the thermospray interface[23–26], many classes of compounds which previously were difficult to analyse by MS can now successfully be analyzed by direct HPLC–MS. Such compounds include those which are highly polar, non-volatile, or thermally labile, such as the phytoestrogens. MS analysis of phytoestrogens in biological fluids has previously necessitated extraction, hydrolysis, purification, and the preparation of volatile derivatives, suitable for introduction into the mass spectrometer via the gas chromatographic outlet[8,10,27,28], techniques which are time consuming.

HPLC–thermospray MS was investigated for its potential to identify individual phytoestrogens in the HPLC effluent under the conditions used here. For all compounds tested the best ionization was achieved at or about a vaporiser temperature of 135°C and a jet-block temperature of 215°C, when the flow-rate was 1 ml/min. Fig. 5 illustrates the total ion current chromatogram, obtained following continuous scanning over the mass range 110–300 *m/z* for a mixture of the five phytoestrogen standards. With the exception of coumestrol, the sensitivity of this technique was comparable to HPLC with UV detection, and no significant loss in chromatographic resolution was observed as a result of interfacing the column with the mass spectrometer. The mass spectra generated in the thermospray ionization process (Fig. 6) were characterized by intense protonated molecular ions, $[MH^+]$, for all of these compounds, and this soft ionization method yielded no significant fragmentation of the molecule.

Since most of the ionization resides in a single ion, selected ion monitoring of the $[MH^+]$ for each phytoestrogen affords a more specific method of detecting these

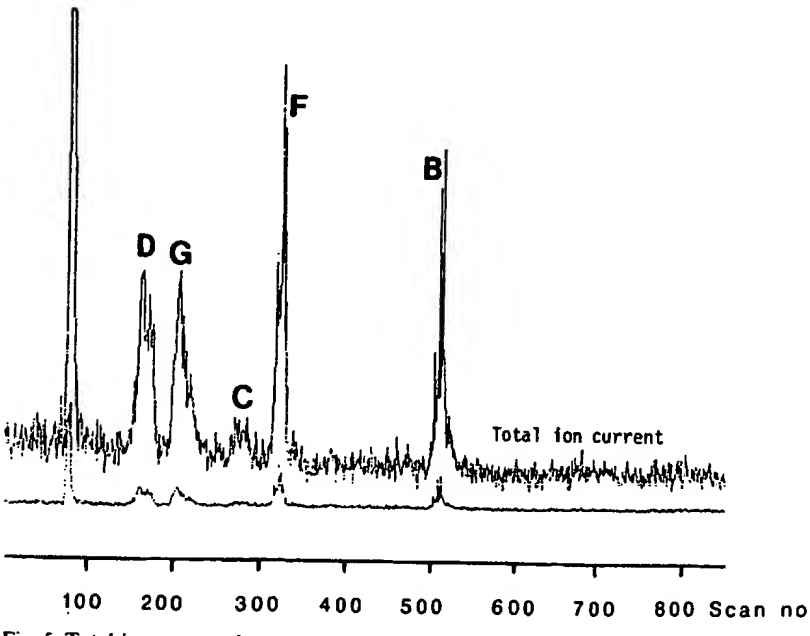
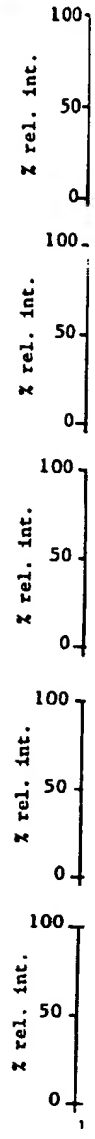


Fig. 5. Total-ion current chromatograms obtained for the HPLC–thermospray MS analysis of a mixture of the phytoestrogen standards listed in Fig. 2.



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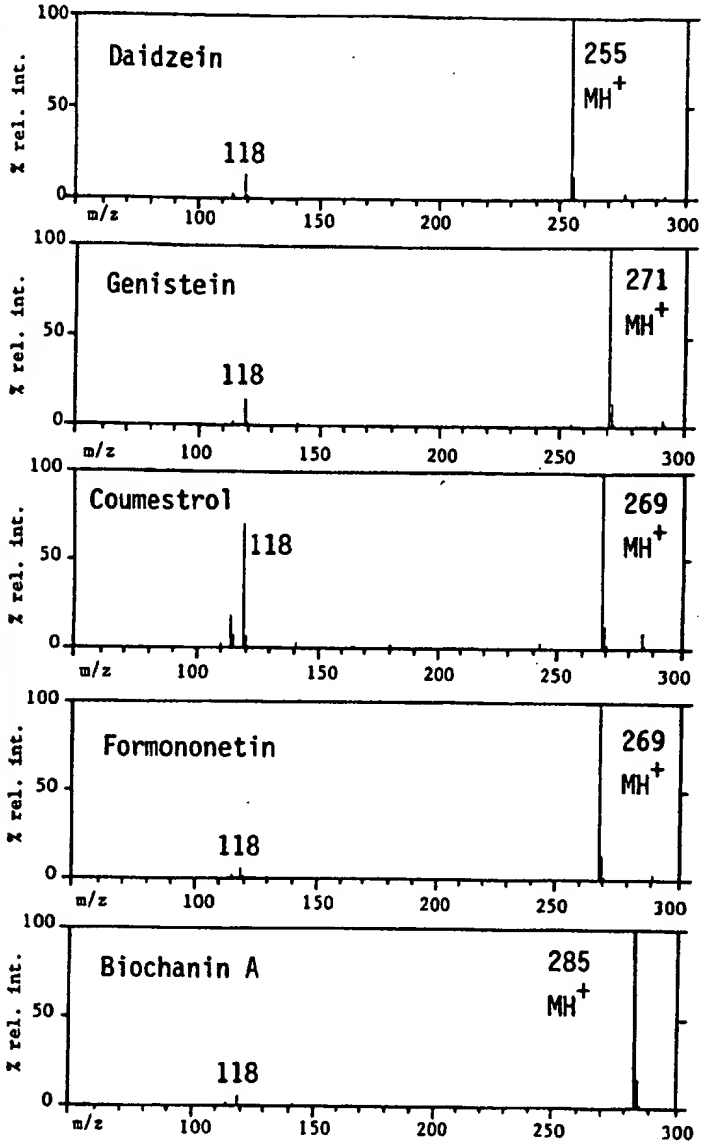

Fig. 6. Mass spectra obtained by thermospray ionization, during HPLC–MS analysis of authentic standards of phytoestrogens.

compounds with a 100-fold improvement in sensitivity over the scanning mode or UV detection alone. Furthermore, we suggest that these compounds would be ideally suited to HPLC–MS–MS detection, where, after focusing the [MH^+] ion, collision-induced dissociation would yield fragmentation specific for each compound, thereby assisting structural elucidation of these and unknown phytoestrogens or metabolites, separated by HPLC. This approach is under evaluation.

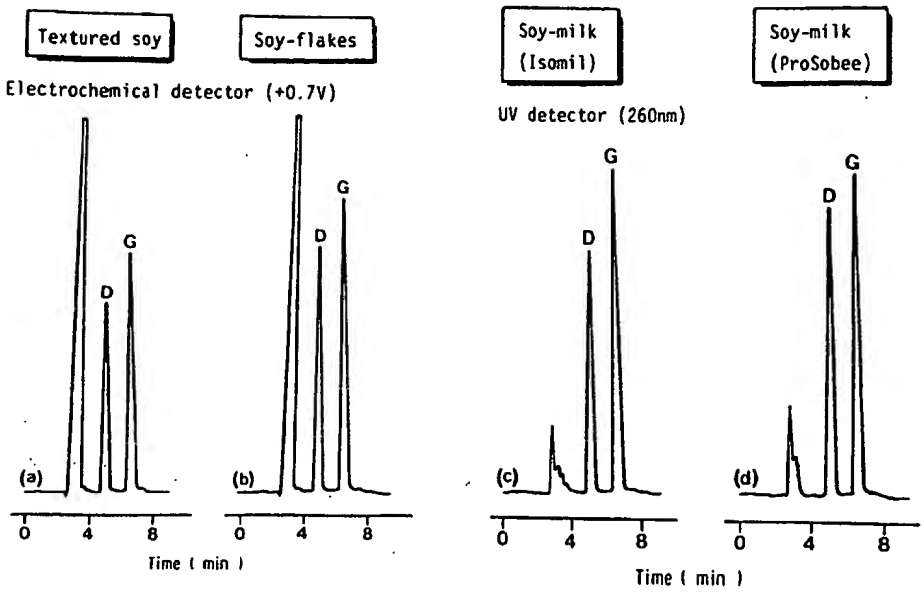

Fig. 7. HPLC profiles of phytoestrogens isolated from samples of soybean products. Both ED and UV detection are illustrated to demonstrate their applicability.

Analysis of soy protein products

Since the phytoestrogens exist in plants mainly as glycoside conjugates[1,2,17,29] or in biological fluids from man and animals as glucuronide or sulphate conjugates[8,10,27], hydrolysis of the conjugate moiety is required prior to HPLC analysis. A general scheme for the analysis of diets or biological fluids was therefore developed to include hydrolysis with glucosidase and/or glucuronidase–sulfatase. Where pure soy protein preparations are to be analyzed, the latter step is unnecessary, but with animal tissues or fluids this step should be considered essential[30]. Ideally, it would be better to develop a system for the direct analysis of the intact conjugates, but at this time the lack of readily available conjugated phytoestrogen standards makes this difficult.

Fig. 7 shows the HPLC analysis for textured soy and soy-flakes by ED and for the soy-milk formulae, Isomil and ProSobee, by UV detection. In all of these soybean products, daidzein and genistein were the only phytoestrogens detected, and

TABLE I

CONCENTRATIONS OF PHYTOESTROGENS IN SOY-BASED PRODUCTS DETERMINED BY HPLC

Soy product	*Daidzein (μg/g)*	*Genistein (μg/g)*
Textured soy	568	568
Soy flake	221	280
Soy-milk formula (ProSobee)	17.1	21.8
Soy-milk formula (Isomil)	19.1	22.6

their concentrations are indicated in Table I. Confirmation of the peaks in each product was made from the mass spectra, obtained by HPLC–thermospray MS, which were identical to those of the authentic compounds.

In earlier reports of the phytoestrogen content of soybean products, daidzein and genistein were the most abundant compounds identified, the latter in slightly higher concentrations[14,15,17–19], but it is evident that there is considerable variability between the different species of soybean and processed products[15].

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The Prostate 22:335–345 (1993)

Genistein and Biochanin A Inhibit the Growth of Human Prostate Cancer Cells but not Epidermal Growth Factor Receptor Tyrosine Autophosphorylation

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The effect of the isoflavones, genistein, daidzein, and biochanin A on the growth of the LNCaP and DU-145 human prostate cancer cell lines has been examined. Genistein and biochanin A, but not daidzein, inhibit both serum and EGF-stimulated growth of LNCaP and DU-145 cells (IC_{50} values from 8.0 to 27 μg/ml for serum and 4.3 to 15 μg/ml for EGF), but have no significant effect of the EGF receptor tyrosine autophosphorylation. In contrast, tyrphostin 25, a specific EGF receptor tyrosine kinase inhibitor, inhibits EGF-stimulated growth and EGF receptor tyrosine autophosphorylation in these whole cells, but does not inhibit serum-stimulated growth. These data suggest that the mechanism of action of genistein and biochanin A does not depend on inhibition of EGF receptor tyrosine autophosphorylation, but on a more distal event in the EGF receptor-mediated signal transduction cascade.

Key words: isoflavones, urogastrone, phosphotyrosine, protein tyrosine kinase

INTRODUCTION

Asians have a markedly lower risk of the hormone-dependent breast and prostate cancers than their American counterparts [1]. However, Asian immigrants to the United States rapidly assume the risk of prostatic cancer seen in U.S.-born males [2]. On the other hand, latent (microscopic) prostatic cancer is commonly found, with similar frequency, in many countries and ethnic groups [3]. These data suggest that environmental factors, especially diet, affect promotional events leading to prostatic cancer and its metastasis, rather than the formation of pre-cancerous lesions in the prostate. An important difference in the diets of Asians and Americans is the consumption of soy-based foodstuffs by Asians. Epidemiologic studies suggest that increased soy consumption is associated with a decreased risk of several cancers (Harvey, Messina, and Barnes, unpublished observations).

We have recently demonstrated that inclusion of soybeans in the diet reduces the number of mammary tumors in rat models of breast cancer [4]. We have also shown

Received for publication October 15, 1992; accepted January 11, 1993.

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that genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7,4′-dihydroxyisoflavone), isoflavones isolated from soy, and biochanin A, the 4-methoxy derivative of genistein, inhibit the proliferative growth of human breast cancer lines in culture, independently of the presence of the estrogen receptor [5]. These data suggest that isoflavones in soy do not act as estrogen agonists or antagonists in vivo, as has been previously suggested [4,6], but act instead through other mechanisms.

Following the observation of Akiyama et al. [7] that genistein is a potent in vitro inhibitor of epidermal growth factor receptor (EGF-R) tyrosine autophosphorylation, several investigators have reported that genistein inhibits the action of other tyrosine kinases involved in signal transduction; these include the platelet derived growth factor receptor [8], c-kit [9], src and other src-family kinases [7,10], $p110^{gag\text{-}fps}$ [7], and $p210^{bcr/abl}$ [11]. This has led to the hypothesis [5] that genistein exerts its inhibitory action on the growth of tumor cells by interfering with the tyrosine kinase activity of activated growth factor receptors and cytoplasmic tyrosine kinases, which are critical for the transduction of mitogenic signals.

The human prostatic cancer cell lines, DU-145 and LNCaP, secrete both EGF and TGF-α (transforming growth factor alpha) [12,13], express functional EGF-R [13,14], and are growth-stimulated by EGF in culture [13,14]. Prostatic cancer cell growth may, therefore, be partially regulated through the EGF-R mediated signal transduction cascade by endogenously produced EGF/TGF-α. Since the mitogenic action of EGF/TGF-α is absolutely dependent on the tyrosine kinase activity of the EGF-R [15], agents that interfere with this kinase activity may be effective inhibitors of prostate cancer growth.

In the present study, we have investigated whether genistein, daidzein, and biochanin A inhibit the serum and EGF-stimulated growth of DU-145 (androgen receptor negative; AR−) and LNCaP (AR+) prostatic cancer cell lines. To explore their inhibitory mechanism of action on cell growth, we examined the effect of isoflavones on the in vivo tyrosine phosphorylation state of the EGF-R. We used this as an indicator to determine if the isoflavones inhibit the tyrosine kinase activity of the EGF-R in vivo. To assess the specificity of genistein and biochanin A against the EGF-R, these results were compared to those obtained with the EGF-R tyrosine kinase selective inhibitor, tyrphostin 25 [16]. This compound specifically inhibits the tyrosine kinase activity of the EGF-R at concentrations several orders of magnitude lower than those that inhibit the tyrosine kinase activity of the insulin receptor [16].

MATERIALS AND METHODS

Materials

3,3′-diaminobenzidine and tyrphostin 25 were obtained from Gibco/BRL (Gaithersburg, MD). Biochanin A was from Aldrich Chemical Co (Milwaukee, WI). Protease inhibitors were from Boehringer Mannheim (Indianapolis, IN). Anti-EGF-R monoclonal antibody (B1D8) was a gift of Dr. Jeff Kudlow (Division of Endocrinology, University of Alabama at Birmingham), polyclonal anti-phosphotyrosine antibody was from Upstate Biotechnology Incorporated (Lake Placid, NY), and goat anti-rabbit IgG antibody was from Kigergaurd and Perry (Gaithersburg, MD). Pansorbin (10%) was from Calbiochem (La Jolla, CA). Other materials were obtained as described previously [5].

Cell Culture

LNCaP and DU-145 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI 1640 medium supplemented with 7% (v/v) fetal-bovine serum and antibiotics (50 μg/ml gentamycin). Cells were cultured as a monolayer (passed every 6–7 days) in a 95% air/5% CO_2 water-saturated atmosphere.

Isoflavone Preparation

Genistein and diadzein were isolated from soy molasses as described previously [5].

Viability Assay

Cytotoxicity of the isoflavones was determined by the MTT assay as described [5], with the following modifications. Cells were plated at 3,000 or 5,000 cells/well in 96-well microtiter plates in serum-containing media and allowed to attach for 24 h. For serum stimulation, drugs (in 100% DMSO; final DMSO concentration in wells, 1% v/v) were added and incubation was continued for an additional 4 days. For EGF-stimulation, serum-containing media was removed and replaced with serum-free, phenol red-free RPMI 1640 and the cells quiesced for 2 days. The serum-free RPMI 1640 was replaced with RPMI ITS-BSA [RPMI 1640 + insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenous acid (6.25 ng/ml), and bovine serum albumin (1.25 mg/ml)]. Drugs were added 15 min prior to the addition of EGF, and incubation continued for 4 days; for incubation with tyrphostin 25, fresh media was added every 48 h.

Cell Lysis and Immunoprecipitation

Cells were grown in 100 cm dishes in RPMI-1640 medium supplemented with 7% (v/v) fetal-bovine serum to 70–80% confluency. Cells were then quiesced for two days in serum-free medium as described above. Medium was replaced by RPMI ITS-BSA medium containing the test compound, and the cells incubated for 15 min at 37°C. Plates were cooled at 4°C for 20 min. EGF was added and incubation continued at 4°C for a further 20 min. The cold RPMI ITS-BSA medium was replaced by fresh medium containing the test compound, and 1 mM Na_3VO_4 pre-warmed to 37°C, and the plates incubated at 37°C for 5 min. Cells were lysed by the addition of 200 μl ice-cold NP-40 buffer (1% NP-40, 150 mM NaCl, Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonylflouride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 mM EDTA, and 1 mM Na_3VO_4). Subsequent steps were performed at 4°C. The plates were rocked for 20 min and cell lysates were scraped into microfuge tubes. The supernatant was passed through a 21 gauge needle 8 times to shear DNA and centrifuged for 10 min at 10,000 × g. Protein concentrations were determined by the Lowry method [17]. EGF-R (100–200 μg lysate protein) was immunoprecipitated from cell lysates by incubation with 1 μM BID8 antibody for 1 h. Immune complexes were collected by a 30 min incubation with Pansorbin (100 μl/ml) and washed 4 times with NP-40 buffer. Immunoprecipitated proteins were resolved on a 7.5% (w/v) SDS-PAGE gel and transferred to nitrocellulose. Tyrosine phosphorylated EGF-R was detected using a polyclonal anti-phosphotyrosine antibody and visualized using a goat anti-rabbit horse radish peroxidase conjugated antibody as described [18].

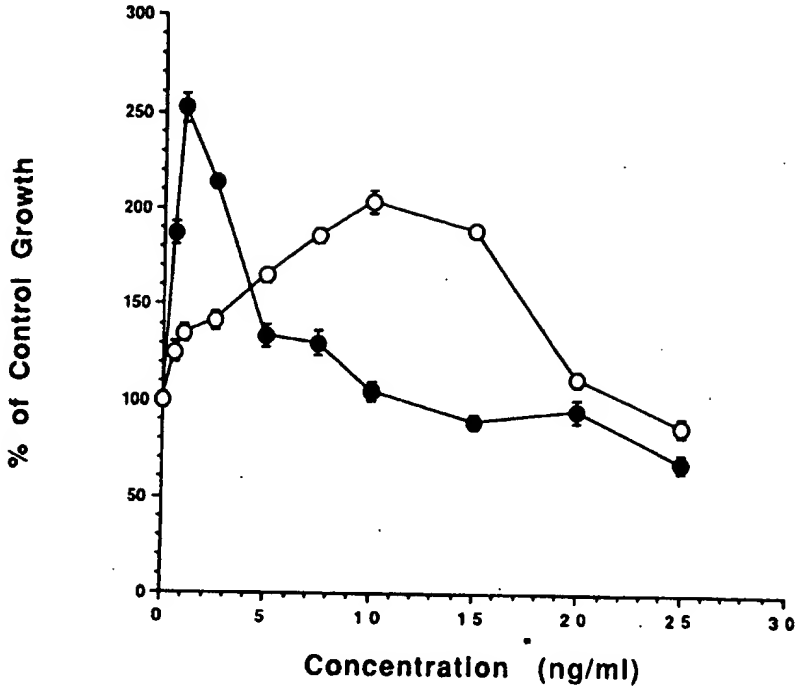

Fig. 1. EGF stimulates the growth of LNCaP (●) and DU-145 (○) human prostate cancer cells. Cells were plated at 5,000 cells/well in RPMI 1640 + 7% FBS and allowed to attach for 24 h. After 48 h quiescence in serum-free RPMI 1640, EGF was added at the indicated concentrations and the cells were incubated for an additional 4 days in RPMI 1640 + ITS-BSA. Cell growth is expressed as a percentage (mean ± standard deviation) of control cells receiving no EGF.

Western blots were subsequently quantitated by reflectance scanning to determine relative inhibition of EGF-R tyrosine phosphorylation.

RESULTS

Effects of Serum and EGF on Growth of LNCaP and DU-145 Cells

The results of experiments to confirm the stimulatory effect of EGF on the growth of LNCaP and DU-145 cells are shown in Figure 1. The cells were cultured in serum-free RPMI 1640 supplemented with ITS-BSA. EGF stimulation was maximal for LNCaP cells at 1 ng/ml and for DU-145 cells at 10 ng/ml. These concentrations were used in all subsequent EGF-stimulated growth experiments.

Inhibition of Serum and EGF-Stimulated Growth by Genistein, Biochanin A, and Tyrphostin

Cytotoxicity of the compounds was determined by the MTT assay following exposure of cells, grown in serum- or in ITS-BSA containing media, to drugs for 4 days. Biochanin A was the most potent inhibitor of serum-stimulated growth with IC_{50} values of 8.0 and 9.0 μg/ml for LNCaP and DU-145 cells, respectively (Fig. 2a,b). Genistein also inhibited the serum-stimulated growth, but at slightly higher

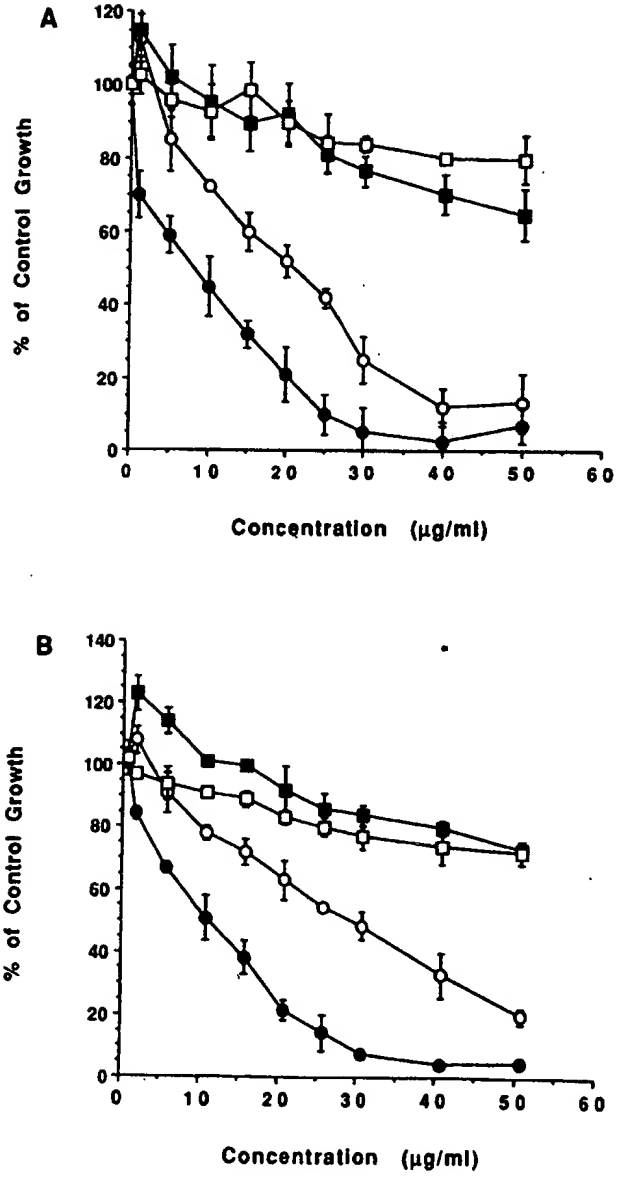

Fig. 2. Inhibition of serum-stimulated growth of LNCaP (A) and DU-145 (B) cells by genistein (○), biochanin A (●), daidzein (■), and tyrphostin (□). Cells were plated at 3,000 cells/well as described in Materials and Methods. Drugs were added at the indicated concentrations on day 2 and incubated in the presence of 7% serum for 4 days. Cell growth is expressed as a percentage (mean ± standard deviation) of control cells receiving DMSO vehicle.

doses (22 μg/ml for LNCaP and 27 μg/ml for DU-145 cells; Fig. 2a,b). In contrast, daidzein and tyrphostin did not inhibit serum-stimulated growth at concentrations up to 50 and 40 μg/ml, respectively (Fig. 2a,b).

In the case of EGF-stimulated growth, genistein, biochanin A, and tyrphostin

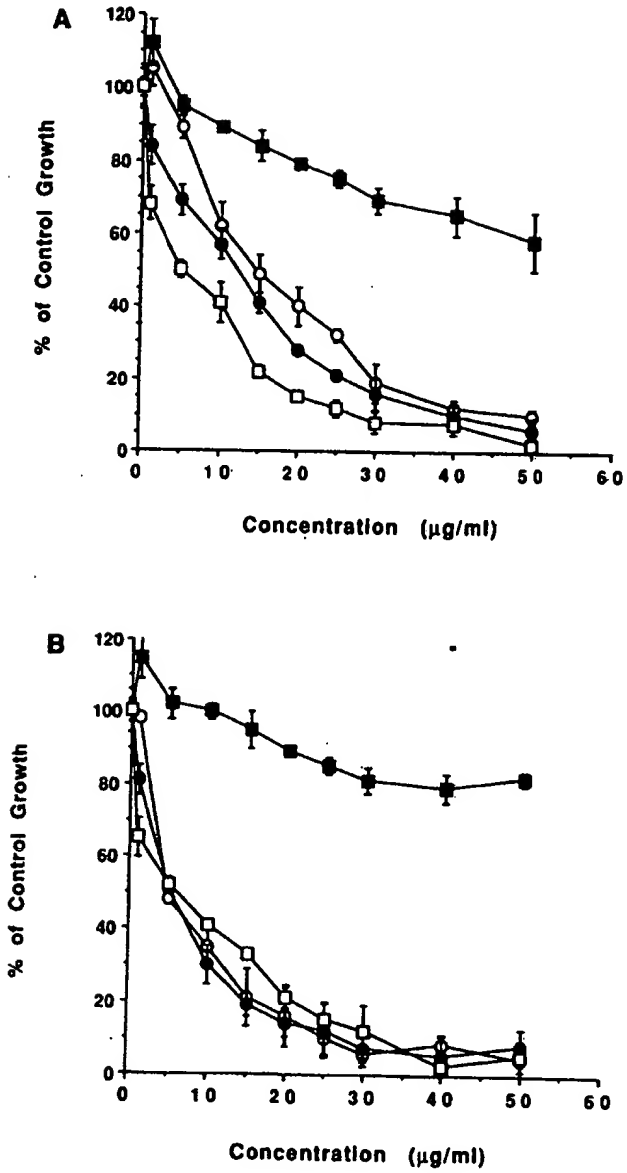

Fig. 3. Inhibition of EGF-stimulated growth of LNCaP (A) and DU-145 (B) cells by genistein (○), biochanin A (●), daidzein (■), and tyrphostin (□). Cells were plated at 5,000 cells/well as described in Materials and Methods. Drugs were added at the indicated concentrations on day 4 and incubated in the presence of EGF (1 ng/ml for LNCaP and 10 ng/ml for DU-145 cells) for 4 days. Cell growth is expressed as a percentage (mean ± standard deviation) of control cells receiving DMSO vehicle.

were each potent inhibitors. The IC_{50} values for tyrphostin on EGF-stimulated growth were 5.2 and 6.0 μg/ml for LNCaP and DU-145 cells, respectively (Fig. 3a,b). Genistein and biochanin A inhibited EGF-stimulated growth, with IC_{50} values of 15 and 13 μg/ml for LNCaP cells, respectively (Fig. 3a), and 4.3 and 5.8 μg/ml for

DU-145 cells, respectively (Fig. 3b). Again, daidzein did not inhibit cell growth at concentrations up to 50 μg/ml (Fig. 3a,b).

Effect of Genistein, Biochanin A, and Tyrphostin on In Vivo EGF-R Tyrosine Autophosphorylation

Tyrosine phosphorylation of EGF-R was determined by immunopreciptating EGF-R from EGF-stimulated, isoflavone- or tyrphostin-treated human prostate cancer cells as described in Methods. The concentration of EGF used to stimulate the cells in these experiments was adjusted to obtain maximal EGF-R tyrosine phosphorylation in a 5 min incubation (10 μg/ml for LNCaP cells and 50 μg/ml for DU-145 cells, respectively; data not shown).

Tyrphostin, at its IC_{50} value for cell growth (6.0 μg/ml), inhibited in vivo EGF-R tyrosine autophosphorylation in LNCaP and DU-145 cells by 72% and 55%, respectively (Figs. 4a and 4b). In contrast, neither genistein nor biochanin A at 10 μg/ml significantly inhibited EGF-R tyrosine autophosphorylation (Fig. 4a,b). At 50 μg/ml, genistein did reduce EGF-R tyrosine autophosphorylation in DU-145 cells by 50% but was largely without effect in LNCaP cells (8% decrease; Fig. 4a,b). Biochanin A was without effect on both cell lines, even at 50 μg/ml. Since high concentrations of EGF were used to obtain maximal EGF-R tyrosine phosphorylation, an additional experiment was carried out using much lower concentrations of EGF (1 ng/ml). Again, genistein at 10 μg/ml had no significant effect on EGF-R tyrosine autophosphorylation (data not shown).

DISCUSSION

In this study, we have established the following points: (1) the isoflavones, genistein and biochanin A, but not daidzein, inhibit the serum and EGF-stimulated growth of the human prostatic cancer cell lines, LNCaP and DU-145; (2) the EGF-R selective tyrosine kinase inhibitor, tyrphostin, inhibits EGF, but not serum-stimulated growth, of LNCaP and DU-145 cell lines; and (3) tyrphostin, but not genistein or biochanin A, inhibits the EGF-stimulated tyrosine autophosphorylation of EGF-R in vivo.

Since genistein and biochanin A inhibit both serum and EGF-stimulated growth, while tyrphostin inhibits only EGF-stimulated growth, the action of genistein and biochanin A cannot be restricted to inhibition of EGF-R activation. This was confirmed by examination of the tyrosine phosphorylation state of the EGF-R after treatment of the cells with EGF and isoflavones or tyrphostin. Neither genistein or biochanin A inhibited tyrosine autophosphorylation of the EGF-R at 10 μg/ml, a concentration close to their IC_{50} values, while tyrphostin 25 blocked tyrosine autophosphorylation of the EGF-R close to its IC_{50} value. The lack of effect of the isoflavones was not due to overstimulation of the cells with EGF, as genistein did not inhibit EGF-stimulated EGF-R tyrosine autophosphorylation at EGF concentrations as low as 1 ng/ml. From the lack of effect of genistein and biochanin A on EGF-R tyrosine phosphorylation, we conclude that the mechanism of action of genistein and biochanin A is not dependent on inhibition of EGF-R activation. We suggest that genistein and biochanin A exert their inhibitory effect by inhibiting tyrosine kinase events at or after a convergence point in signal transduction pathways, or act through mechanisms other than tyrosine phosphorylation.

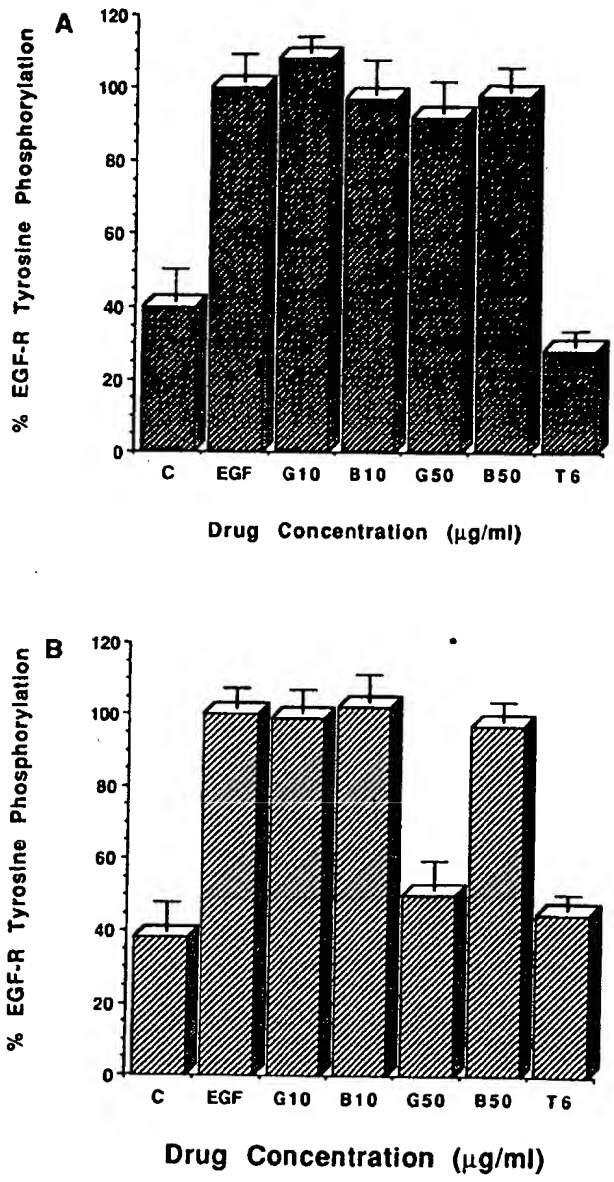

Fig. 4. Inhibition of EGF-R tyrosine autophosphorylation by isoflavone and tyrphostin in LNCaP (A) and DU-145 (B) cells. Cells were incubated in the presence of the indicated drug (G, genistein; B, biochanin A; T, tyrphostin 25; concentration in μg/ml) and lysed as described. EGF-R was immunoprecipitated, separated by SDS-PAGE, blotted, and visualized as described in Materials and Methods. Blots were quantitated by reflectance scanning and results expressed as a percentage (mean ± standard deviation) of EGF-stimulated tyrosine incorporation into EGF-R.

Several investigators have reported data that support the idea that isoflavones do not directly inhibit EGF-R activation. Okura et al. found no correlation between the inhibition of EGF-R tyrosine kinase activity by isoflavones in vitro and reduction of

the in vivo growth of Ha-*ras* transformed NIH-3T3 cells [19]. Additionally, Linnasier et al. found that genistein elicited cytostatic effects in NIH-3T3 cells, without inhibiting the signaling capability of EGF-R [20]. This was consistent with the presence of an alternative, more sensitive isoflavone target in the cell, which these authors suggested was ribosomal S-6 kinase. S-6 kinase is postulated to be activated by MAP kinase [21], a key signal transduction intermediate activated by many diverse types of stimuli [21 and references therein], which requires tyrosine phosphorylation for maximal activity [22]. More importantly, genistein has been shown to be an inhibitor of other cytoplasmic tyrosine kinases, such as src [7] and $p210^{bcr\text{-}abl}$ [11], which demonstrate that genistein can inhibit tyrosine phosphorylation events distal to membrane-bound growth factor receptors.

An alternate explanation could be that the isoflavones do not inhibit tyrosine phosphorylation events at all, but regulate other events critical for cell growth and proliferation. For example, the isoflavone psi-tectorigenin (8-methoxygenistein) inhibited phosphatidylinositol turnover in A-431 cells without inhibiting EGF-R tyrosine kinase activity [23]. Furthermore, Makisshima et al. [24] recently reported that psi-tectorigenin and genistein induce the differentiation of ML-1 and HL-60 cells (psi-tectorigenin > genistein) to a greater extent than the more potent tyrosine kinase inhibitor methyl 2,5-dihydroxycinamate, which suggests that the differentiation inducing effect of genistein may be due to inhibition of phosphatidylinositol turnover, rather than inhibition of tyrosine kinase activity [24]. Other investigators have suggested that genistein may exert its effect through induction of DNA strand breakage [25] or inhibition of DNA topoisomerase II [26]. Additionally, the isoflavones may act through an as yet unidentified pathway.

The data from the present study also demonstrate that a functioning AR system is not essential for isoflavones to inhibit the growth of human prostate cancer cells. However, in LNCaP cells the IC_{50} values for genistein and biochanin A on EGF-stimulated growth are 2- to 3-fold higher than the IC_{50} values for the compounds in DU-145 cells. It has previously been shown that the AR from LNCaP cells is mutated, and this mutation relaxes the specificity of the AR and allows ligands other than androgen to activate the AR [27]. It is also known that activated AR can increase the number of EGF-R on the cell surface, thus making the cell more sensitive to EGF/TGF-α stimulation, which leads to increased cell growth [28]. It is possible that genistein and biochanin A are weak binders of AR in LNCaP cells, and this partial activation of AR results in increased signal transduction through the EGF-R. This, in turn, could decrease the effectiveness of genistein and biochanin A, which would result in higher IC_{50} values in EGF-stimulated growth assays and lower inhibitory activity of these compounds against EGF-R tyrosine autophosphorylation.

CONCLUSIONS

The results obtained from this study show that genistein and biochanin A inhibit the serum and EGF-stimulated growth of human prostate cancer cell in culture, without completely blocking the initial events of EGF-R activation. In addition, this study supports the notion that genistein is one of the active anti-cancer agents in soy. Since genistein is essentially absent from the U.S. diet [29], in contrast to the Asian diet (Messina, unpublished observations), soy may be an important nutritional factor which is responsible for the low rate of prostate cancer in Asian men.

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